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CHEMICAL INVESTIGATION OF THE FUNGUS'S ETHANOLIC EXTRACT *PISOLITHUS TINCTORIUS* AND EVALUATING ITS IMPACT ON *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

Scientific research today is dedicated to the discovery of new molecules with anti-inflammatory properties and new antimicrobials to combat antibiotic resistance, all to ensure the survival of humanity in the face of the need for new treatments in health. *Pisolithus* (Basidiomycota) is a widely distributed genus of fungi which occurs in forest habitats and has been identified as an ectomycorrhizal fungus interacting with a wide range of host plants to stimulate their development and protect against biotic and abiotic stresses. In addition to its ecological applications, the ethanol extract of the fungus *Pisolithus tinctorius* has recently been found to contain bioactive compounds which inhibit the growth of some species of bacteria. Bioactive substances with therapeutic potential are produced by *pisolithus.* Given the broad spectrum of antibacterial activities of *Pisolithus* fungus, The ethanolic extract of *Pisolithus tinctorius* exhibited no inhibitory effect on *Klebsiella pneumoniae*. GC-MS analysis of the chemical composition supports this, showing that saturated alkanes lacking functional groups accounted for nearly 86% of the total peak area. Consequently, the SI values were almost the same for each of these peaks among the first five hits. At a retention time of 4.880 min, dodecane recorded 97, pentadecane 96, tetradecane 95, and tridecane 95. The standard minimum match requirement is 800 of 999, or roughly SI 80 on our instrument, according to NIST.

Keywords: *Pisolithus tinctorius*, Ethanolic Extract, Tetradecane, Dodecane, *Klebsiella pneumoniae*

1 INTRODUCTION

Klebsiella pneumoniae is an encapsulated, Gram-negative, opportunistic pathogen, particularly associated with severe nosocomial infections such as pneumonia, urinary tract infection, bloodstream infection and septicemia. The clinical management of *K. pneumoniae* has been complicated by the emergence of multidrug-resistant (MDR) and extended-spectrum beta-lactamase (ESBL) producing organisms, which have spread rapidly. In addition to strains, there are also carbapenem-resistant *Klebsiella pneumoniae* (CRKP). Thus, there is an urgent need for identifying new antimicrobial agents which have novel mechanisms of action to overcome the existing resistance mechanisms [1]. The characteristic fruiting bodies (Basidiocarps) of *Pisolithus* species, along with their extensive distribution on various continents in

various settings, are indications of their characteristic fruiting bodies. The basidiocarps of *Pisolithus* are large, puffball-shaped structures, often buried in the soil. Besides the basidiocarps, *Pisolithus* consists of spores that are generated by the basidiocarps and the hyphae, which are thread-like structures that make up the fungus' mycelium [2]. One of *Pisolithus's* most unique features is that its species are ectomycorrhizal fungi that are associated with plants, particularly trees. There are 19 species of *Pisolithus* which infect more than 50 host plants. Mycorrhizal association is a mutualistic association [3]. The fungus helps the plant take up nutrients, especially phosphorus , and the plant supplies the fungus with carbohydrates that it makes by photosynthesis . In this symbiotic association, it enhances the growth, and can withstand biotic and abiotic stressors [4]. The positive aspects of *Pisolithus* species have been introduced into forestry management strategies to create healthier ecosystems, and improve the long-term sustainable productivity of forestry plantations [5]. Of the various species of *Pisolithus*, *P. arhizus* is the most studied.

In addition to their ecological role and their use in forestry, the bioactive compounds produced by *Pisolithus* species have attracted the interest of researchers have identified substances with antimicrobial, anticancer and antioxidant properties. The results provide the basis for further studies on the medicinal potential of compounds from *Pisolithus* [6].

The challenges faced by contemporary medical research are pushing the field toward innovative solutions. The discovery and development of new antimicrobial products is of utmost importance in addressing the increasing problem of drug resistance [7]. In addition, the incessant search for new natural molecules in cancer therapy reflects the critical need for transformative approaches to improve therapeutic outcomes in the ongoing fight against cancer [8,9]. At the same time, the quest for antioxidant molecules active against inflammation-related diseases is a response to widespread health concerns [10].

In this study, considering the transversal biological activity of *Pisolithus*, the aim of this study is to present an exhaustive study on the antimicrobial potential of these fungi. The focus is also on highlighting the bioactive compounds isolated from Pisolithus with the aim to catalyze further research for exploring innovative uses for these ubiquitous and widely distributed fungi.

2 MATERIALS AND METHODS

2.1 Preparation of the Mushrooms

The fruiting bodies of the mushroom *Pisolithus tinctorius* were obtained from the city of Baquba. The fruiting bodies were collected from a single location to minimize the impact of environmental variations among the samples. In October 2024, they were identified morphologically, microscopically, and based on measurements according to the description in [11]

2.2 Preparation of the Aqueous Extract:

The aqueous extract of the mushroom was prepared by weighing 10 g of dry mushroom powder, adding 100 mL of distilled water, and allowing it to stand for 24 hours in the refrigerator. It was then placed on a magnetic stirrer for an additional 24 hours to extract the maximum amount of active compounds. The extract was filtered through several layers of gauze to remove large particles of the fungal powder, followed by filtration using Watman No. 1 filter paper, and then stored in the refrigerator in airtight containers until use.

2.3 Extraction Using Aqueous Ethanol (70%)

50 g of dry mushroom powder was weighed and placed in a Sechslet flask; aqueous ethanol (70%) was then added to the flask. Extraction was carried out for 4 hours under reflux, after which the extract was concentrated using a rotary vacuum evaporator; the resulting extract was then collected and weighed to determine the extraction yield [12]

2.4 Preparation of the Bacterial Culture

A Klebsiella pneumoniae isolate was obtained from the laboratories of Tikrit Hospital, where it was isolated from patients with severe burns. It was purified, identified microscopically, and identified using the VITEC system. Only one isolate was used, which was cultured on Neutron liquid medium to obtain a bacterial suspension with a concentration of approximately: (1.5×10^8 cells/mL) to match the McFarland standard.

2.5 Antibacterial Activity Test

2.5.1 Agar Well Diffusion Method

The method [13] was adopted as follows: 25 mL of nutrient agar was poured into sterile Petri dishes and allowed to solidify. The plates were inoculated by adding 0.1 mL of the bacterial suspension, then spread using a sterile spreader. The plates were left to dry at room temperature. Wells with a diameter of 5 mm were made using a sterile cork borer, with 3 wells in each plate.

(0.1)mL of various concentrations of fungal extracts was added to the wells using a sterile micropipette. Three replicates were performed for each treatment. The plates were incubated at (37°C) for (24–48) hours. The efficacy of all extracts was tested at the same concentrations (25, 50, 75, 100) mg/mL, prepared by dilution. After the incubation period, the results were observed by measuring the inhibition zones around the wells. Levofloxacin 5 mg antibiotic discs were used for comparison with the extracts. The samples were analyzed using a Shimadzu GCMS-QP2010 Plus instrument manufactured in Japan.

The samples were analyzed using a Shimadzu (Japan) gas chromatography–mass spectrometry (GC-MS) system, model GC-MS-QP2010 Plus. The injector was set to split mode at a temperature of 150°C, with a sample volume of 1 microliter. A DB-5ms capillary column with a membrane thickness of 0.25 micrometers and a length of 30 meters was used for separation. The carrier gas was helium (99.999% purity) at a column flow rate of 1.27 mL/min (and a total flow rate of 16.9 - mL/min). The oven temperature program began at 100°C and was maintained for one minute, after which the temperature was raised at a rate of 8 °C/min until reaching 150 °C and held for 4 minutes; then the temperature was raised again at a rate of 12.5 °C/min to 250 °C and held at this temperature for one minute, resulting in a total program duration of 20.25 minutes. Mass spectra were recorded in the range (m/z 30–900) at an ionization energy of 72 eV.

Mass spectra were recorded in the range m/z 900–30 at an ionization energy of 72 eV. The chemical compounds extracted from the studied samples were identified by comparing the resulting mass spectra with those in the libraries available in the instrument's software. (14) that the method is under development and not yet standardized; it varies depending on the nature of the sample components.

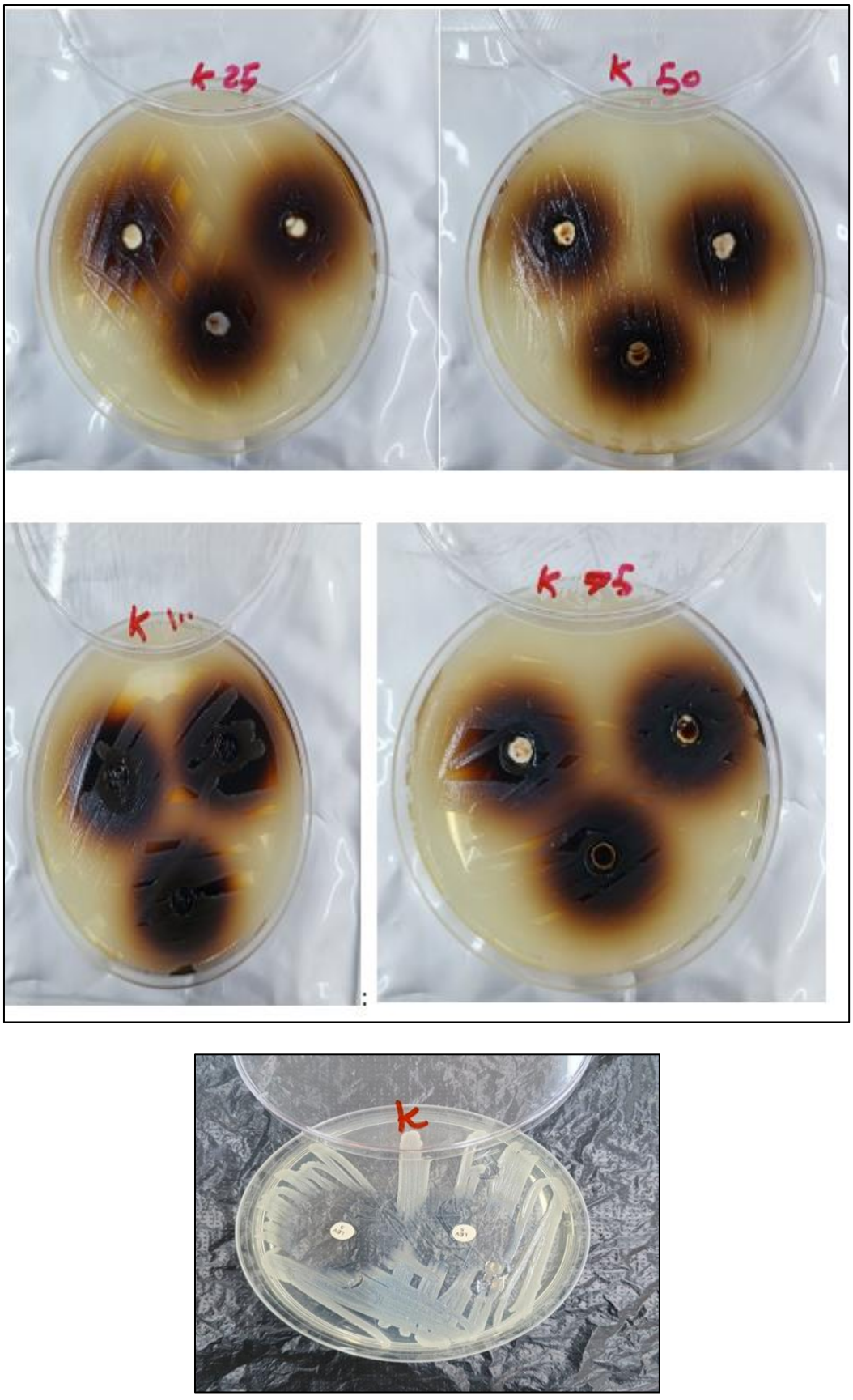


Figure 1: Inhibitory activity of the ethanolic extract *Pisolithus tinctorius* against *Klebsiella pneumoniae*

3 RESULTS

Ethanolic extract of *Pisolithus tinctorius* showed no inhibitory activity against *Klebsiella pneumoniae.* This is in accordance with the chemical composition as indicated by GC-MS analysis, which showed that almost 86% of the total peak area was due to saturated alkanes without functional groups. The chromatogram did not reveal the chemical classes that are known to be biologically active in this fungus [14].

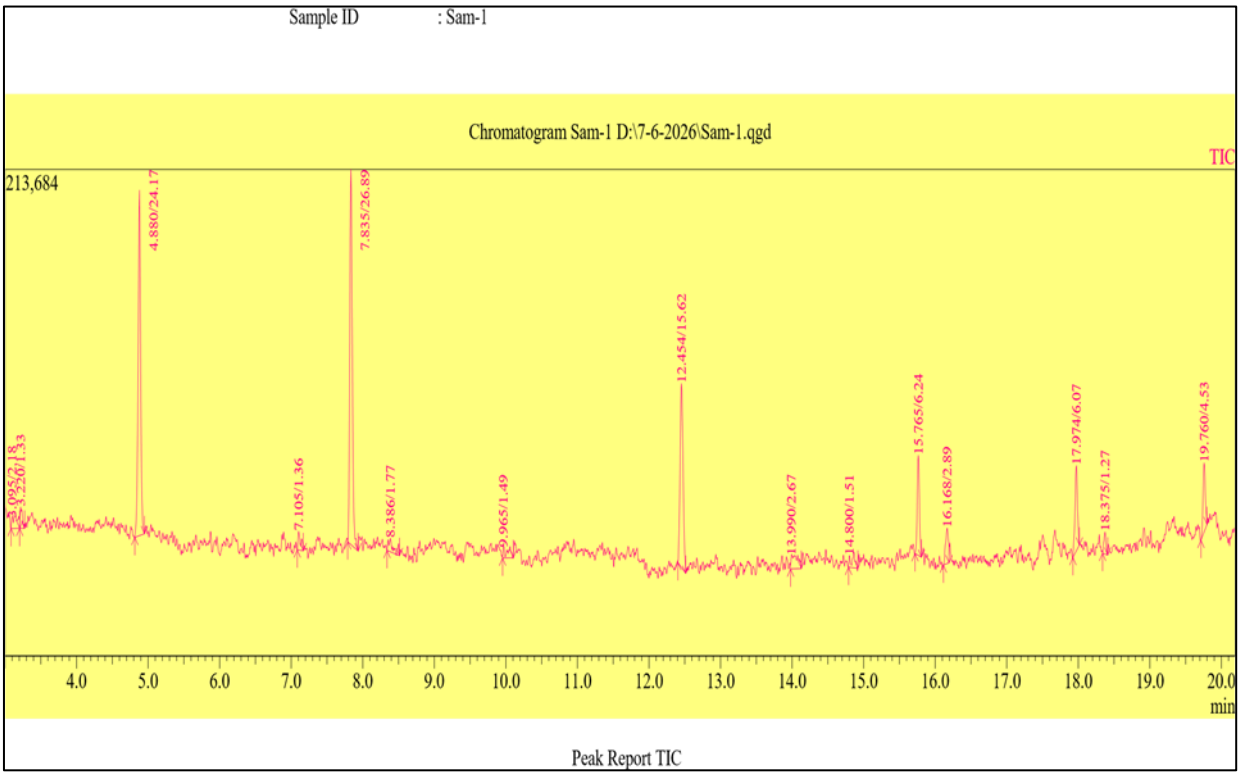


Figure 2: Gas Chromatography–Mass Spectrometry (GC–MS) Chromatogram of the Ethanolic Extract of *Pisolithus tinctorius*

3.1 Chemical composition of the extract as revealed by GC-MS

Within 20 minutes, a chromatogram showed 15 peaks, and three straight-chain alkanes—tetradecane, dodecane, and hexadecane—accounted for the majority of them, comprising 66.7% of the total area. Straight-chain and branched alkanes together made up roughly 86% of the total. The analysis took place at the Central Laboratory of the College of Applied Sciences, University of Samarra, on sample Sam-1, and the compounds were identified using the NIST11 library [15].

Alkanes are saturated hydrocarbons consisting solely of carbon and hydrogen, and they are nonpolar. They are chemically inert, almost insoluble in water, and exhibit properties comparable to those of oils and waxes [16]. Dependability and the boundaries of identification. Although the alkane class is confirmed to be present in this sample, additional validation is required to determine the carbon chain length of each peak. The following considerations clearly demonstrate the definition's shortcomings [17]: The spectra within the homologous series are similar. The fragmentation pattern of straight-chain alkanes is identical (m/z 43, 57, 71, 85, 99), and these compounds also show a distinctive peak at m/z 57 together with a molecular ion that is either weak or missing. As a result, the SI values at each of these peaks were nearly identical for the first five hits. At a retention time of 4.880 min, the values recorded were 97 for dodecane, 96 for pentadecane, 95 for tetradecane, and 95 for tridecane. To settle this question, the Kovats Retention Index must be calculated by injecting a standard alkane mixture covering C8 to C40. In a similar investigation, library matches were shown to be unreliable whenever the probability lay between 70% and 90%, because of overlapping peaks and inadequate resolution [18]. Research looking at how reference materials are prepared from straight-chain alkanes (C10–C16) showed that GC-MS couldn't identify alkane impurities, since these impurities have the same characteristic ions (m/z 43 and 57) as the main compound, and the NIST library didn't give satisfactory matches for their spectra—especially for pure decane, which matched at only 85.9% (a result confirmed by other methods). By comparing the retention times of these impurities with those of a series of straight-chain alkanes analyzed under identical conditions, the number of carbon atoms in the impurities was determined [19]. The standard minimum match requirement is 800 out of 999, which corresponds to roughly SI 80 on our instrument, according to researchers at the

National Institute of Standards and Technology (NIST), the agency responsible for creating the library. In addition, the degree of match on its own is not enough to identify a library**.**

Table 1: Compounds detected by GC-MS in the ethanolic extract (sample Sam-1)

Peak no.	RT (min)	Reported compound	Formula	Area %	SI	Suggested assessment
5	7.835	Tetradecane	C14H30	26.89	98	Strong class identification
3	4.880	Dodecane	C12H26	24.17	97	Strong class identification
8	12.454	Hexadecane	C16H34	15.62	96	Strong class identification
11	15.765	Eicosane	C20H42	6.24	94	Good
13	17.974	Nonadecane, 9-methyl-	C20H42	6.07	92	Long-chain alkane (top hit eicosane, SI 94)
15	19.760	Tetratetracontane	C44H90	4.53	92	Probably a C21–C24 alkane (top hit eicosane, SI 93)
12	16.168	Dodecane, 2-methyl-6-propyl-	C16H34	2.89	80	Branched alkane, accept with caution
4	7.105	Methoxyacetic acid, 6-ethyl-3-octyl ester	C13H26O3	1.36	73	Tentative
6	8.386	4-Amino-2-oxy-furazan-3-carboxylic acid	C3H3N3O4	1.77	47	Unreliable
10	14.800	1H-1,2,4-Triazole-3-carboxylic acid, 5-hydroxy	C3H3N3O3	1.51	55	Unreliable
14	18.375	Methoxyacetic acid, 4-hexadecyl ester	C19H38O3	1.27	46	Unreliable
1, 2, 7, 9	3.095, 3.220, 9.965, 13.990	No hit	—	7.67 (combined)	—	Unidentified

4 DISCUSSION

Given that adding a CH_2 group equates to about 100 units, they maintain that the Kovats retention index, which uses the retention time of linear alkanes as a reference, is essential for confirming identity in complex mixes. The identification is considered tentatively identified compounds when the analyst does not offer confirmation [20]. By normalizing the retention time against a set of straight-chain alkanes, the impact of fluctuating operating circumstances is minimized, allowing results to be compared with retention index databases like NIST and enabling the separation of compounds with similar spectra. members of the same homologous series, for instance [21]. Spectral matching ranked the correct compound first in 68% of the 100 compounds in the NIST collection; accuracy increased to 80% when the spectrum and retention index were combined [8]. Peak 15 (C44, tetratetracontane). Eicosane (SI 93) was the top entry in the search results, followed by C44 (SI 92). Moreover, in a 20-minute run, an alkane with a molecular weight of 618 and a retention index of around 4395 cannot elute just four minutes after eicosane. It should be defined as a long-chain n-alkane since it is probably a C21 to C24 alkane [18].9-methylnonadecane, peak 13. The reported name was derived from the second hit (SI 92), while the first hit was eicosane (SI 94). As a result, it is a C20 alkane, either straight or branched, with an unknown branching position [17]. The reported polar compounds (peaks 6, 10, and 14). Their base peaks happened at m/z 44 and 45, which match the usual background peaks of CO2 or an air leak, and their SI values varied from 46 to 55. As a result, they have no biological activity and shouldn't be considered separate components of the extract. The certainty of identification is reduced by co-eluting peaks and background ions, and this effect increases with decreasing peak height [16]. Methoxyacetic acid esters. These are common false matches that appear for branched alkyl fragments in the NIST collection. They could be classified as unknown aliphatic chemicals [17]. the early peaks (3.0–3.2 min) and the peaks without a match. These most likely relate to the solvent front, column bleed, or backdrop of an instrument. The early peak at 3.0 minutes that corresponds to methylsilane [19] further supports this. likely contamination. The series only includes even-chain lengths (C12, C14, C16, and C20), with C13, C15, and C17 missing. Odd-chain hydrocarbons predominated in 51% of the samples, whereas even-chain hydrocarbons predominated in only 10%,

according to a thorough analysis of 592 biogenic soil samples of non-cyclic hydrocarbons [19]. Methoxyacetic acid esters. These are common false matches that appear for branched alkyl fragments in the NIST collection. They could be described as unknown aliphatic chemicals [17]. The peaks that don't have a match, along with the early peaks (3.0–3.2 min). Most likely, these correspond to the solvent front, column bleed, or instrument background. This is also supported by the early peak at 3.0 min matching methylsilane [19]. Probable contamination. While C13, C15, and C17 are absent, the series contains only even-chain lengths (C12, C14, C16, and C20). A comprehensive review of 592 biogenic soil samples of non-cyclic hydrocarbons found that odd-chain hydrocarbons predominated in 51% of the samples, while even-chain hydrocarbons predominated in only 10% [15]. Nevertheless, the same study did not find that these additives transferred into organic solvents when analyzed by headspace analysis; as a result, this source remains a possibility. Another possible source is spurious peaks arising from carryover of earlier samples in the column. This can be ruled out only by injecting a blank sample of the same ethanol sample. The active compounds found in *P. tinctorius* are polar or have a high molecular weight, and they are not volatile under the GC-MS conditions unless they undergo chemical derivatisation. It works well for volatile and semi-volatile compounds and is most useful for compounds with boiling points below 200 °C, even though it was originally designed for small, volatile, non-polar molecules [16]. As a result, the chromatogram reflects only the volatile fraction of the extract, which is the least biologically relevant portion. Pulvinic acid derivatives are one of the chemical classes that have been reported for this fungus. The yellow and brown colors of the fruiting bodies are mainly due to these pigments, which include norbadione A, badione A and pisoquinone [20]. Norbadione A is a pulvinic acid derivative found in nature that acts as an antioxidant; because it has several carboxylic and enolic groups, it is highly polar and non-volatile. They have been reported to have antifungal activity, inhibiting spore germination of *Truncatella hartigii* at 67 and 71 µg/mL, respectively [18]. Both are carboxylic acids containing a phenolic group, and they cannot be identified by GC-MS without derivatisation [19]. These are triterpenes that belong to the lanostane type. The most important one is pisosterol, which is the main colourless constituent of the fruiting bodies [20], along with pisosteral, 3-epi-pisosterol and lanosterol [21]. These are polyhydroxylated compounds of high molecular weight that require derivatisation in order to pass through a GC column [20]. Before analysis, silylation of these classes with reagents such as BSTFA or MSTFA is required. These reagents swap the active hydrogen in hydroxyl and carboxyl groups for a trimethylsilyl group, which makes the compound more volatile and thermally stable [22]. The bioactive compounds known to be present in the fungus and the reason they were not detected. The compounds responsible for the biological activity in *P. tinctorius* are polar or have high molecular weight, and therefore they cannot be volatilised under GC-MS conditions without chemical derivatization. Consequently, the chromatogram reflects only the volatile portion of the extract, which is the least biologically relevant of all the components [23]. Pulvinic acid derivatives are the chemical classes that have been reported in this fungus. Yellow and brown hues are imparted to the fruit bodies by the principal pigments, which include norbadione A, badione A, and pisoquinone. [24, 25]. Both of them are carboxylic acids containing a phenolic group, and therefore they cannot be detected by GC-MS without prior derivatisation [26]. Triterpenes of the lanostane type. The most important one is pisosterol, the main colourless component of the fruiting body, along with pisosteral, 3-epi-pisosterol, and lanosterol. These compounds have a very high molecular weight and must be derivatized in order to pass through a GC column [27]. For analysis within these classes, samples need to be silylated, for instance with BSTFA or MSTFA. The active hydrogen in hydroxyl and carboxyl groups is replaced with a trimethylsilyl group by these reagents, making the compound more volatile and thermally stable [28]. Gaid et al. [18] detected the carboxylic acid degradation products of the alkanes only after methylation with diazomethane, which converts carboxyl and hydroxyl groups into methyl esters and ethers, respectively. Given that fewer than 2% of known compounds in metabolomics and natural products have experimental mass spectra and even fewer have known retention indices, the fungal metabolites may never have reached the detector and thus would not appear in the NIST11 library [29].↻ Explain why there was no activity against *Klebsiella pneumoniae*. The absence of inhibition has been explained by three overlapping factors that have been proposed: the structural barriers of this bacterium, the limitations of the test method, and the nature of the extract's dominant constituents. [30]. Alkanes are hydrocarbons that lack any hydroxyl, carbonyl, or amine groups. As a result, they will not inhibit target proteins or bind to bacterial enzymes. They are also essentially insoluble in aqueous media, which means they cannot reach an effective concentration around the cell.↻ In fact, these alkanes aren't inhibitors — they're substrates that filamentous fungi can metabolise. These fungi were isolated from oil-polluted soils, and after seven days they had degraded 97% or more of the tetradecane, the main component of our sample, with a significant increase in biomass [31]. The outer membrane. Gram-negative bacteria possess an outer membrane composed largely of lipopolysaccharide (LPS), which is chiefly responsible for blocking the entry of harmful substances and aiding the uptake of nutrients. This is why most natural extracts work against gram-positive bacteria but not against gram-negative ones. [32]. Even the active extracts of this fungus require very high concentrations in order to inhibit *K. pneumoniae*. For multidrug-resistant (MDR) *K. pneumoniae* isolates, the fungus's hydroethanolic extract showed a minimum inhibitory concentration of 5 mg/mL [33]. A lower concentration would therefore not be expected to produce inhibition. Two key points emerge from this comparison. Activity against *K. pneumoniae* was seen only at extremely high concentrations of crude extract (5 mg/mL) [34] or of a purified compound containing a carboxyl group [35]. Second, the present chromatogram did not contain any of the active fatty acid identified in Carmo's study, which may be explained by the fact that free fatty acids must be esterified or silylated before GC-MS analysis [36]. The active compounds isolated from the genus *Pisolithus* are mostly phenolics and triterpenes, as Martins et al. (2024) also reported, and these are absent from the volatile components of the present sample [37].

5 CONCLUSION

This finding did not show significant effectiveness under the conditions of the present study. Therefore, further research is needed to evaluate their efficacy and optimize the conditions required to achieve better results.

However, does imply that the fungus has biological activity; it indicates that its active components were found examined using the proper technique. The 15 chromatographic peaks found in the Sam-1 extract after 20 minutes on the GC-MS with peak areas of 3.7% of the total chromatogram area demonstrated the presence of multiple chemical constituents, mostly hydrocarbons. Straight-chain alkanes (66.7% of the total chromatographic peak area) and branched and straight-chain alkanes (about 86% of the total peak area) were the two major components. From these results, it was noted that the major chemical class in the extract was alkanes. These compounds are similar in structure and have overlapping fragmentation patterns, and identification by NIST11 library matching should be taken as a preliminary identification.

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