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Research article

Antimicrobial characteristics of endophytic *Aspergillus terreus* and acute oral toxicity analysis ☆

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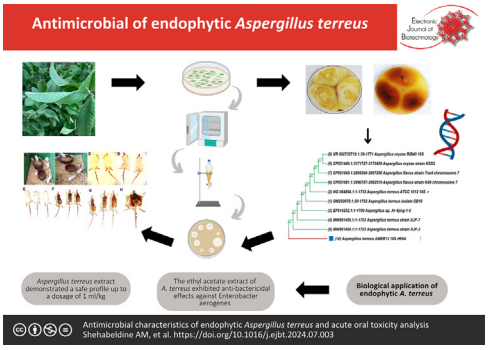
GRAPHICAL ABSTRACT



Antimicrobial of endophytic *Aspergillus terreus*

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ABSTRACT

Background: Endophytic fungi produce biologically robust metabolites suitable for diverse applications, which support the increasing exploration of these fungi. The study aimed to investigate the *in vitro* antimicrobial properties of the metabolites of endophytic fungus *Aspergillus terreus* which isolated from the leaves of *Psidium guajava* plants while the acute oral toxicity was assessed *in vivo*.
Results: A. terreus (OR125572) was successfully isolated for the first time from *P. guajava*. The ethyl acetate extract of *A. terreus* exhibited antibactericidal effects against *Enterobacter aerogenes* with a minimum inhibitory concentration of 18.75 mg/ml in contrast to 75.00 ± 0.0, 37.50, and 37.50 mg/ml, respectively against *Staphylococcus aureus, Escherichia coli, and Bacillus sphaericus* were 75.00 ± 0.0, 37.50, and 37.50 mg/ml, respectively. Analysis of the crude extract obtained from *A. terreus* by GC-MS revealed a total of 32 distinct compounds. The major components included 1,2-benzenedicarboxylic acid, di-iso-octyl ester; hexyl oxecan-2-one and phenol. The acute oral toxicity study found no symptoms of toxicity and no mortality until the 14th d, suggesting that the LD_{50} value of *A. terreus* extract might exceed 1 ml/kg. The group receiving 0.5 ml/kg of *A. terreus* extract experienced a 42.9% increase in body weight, while administration of varying dosages resulted in a significant reduction in MCV levels. There was also a significant increase in the proportion of monocytes across all treated groups.

☆ Audio abstract available in Supplementary material.

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Conclusions: The study demonstrated the potential of *A. terreus* as a source of antimicrobial and other bioactive compounds, with relatively low acute toxicity.

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1. Introduction

The long-term use of traditional antibiotics and antifungals has led to the emergence of strains of bacteria and fungi pathogenic to humans that are resistant to these treatments. This dilemma is considered one of the most significant problems currently facing scientists. Therefore, scientists are recently focusing on producing biological alternatives to traditional fungal and bacterial inhibitors [1]. Endophytic fungi are a promising source of bioactive compounds in various fields, including medical, agricultural, and industrial [2,3]. In this regard, some points must be considered when isolating endophytes, containing the type of plant used, the plant parts used, and the isolation and identification methods [4]. Guava (*Psidium guajava* L.) is a renowned tropical tree planted extensively in tropical regions for its fruit [5]. Due to its pharmacological qualities, this plant is used as both a food source and a traditional medicinal remedy. The *P. guajava* plant was chosen because previous studies have proven its effectiveness in producing biologically active substances. The leaves of *P. guajava* contain a variety of phytochemical compounds that contribute to its medicinal properties including ascorbic acid, pectin, and antioxidants, and areis particularly beneficial for prostate and heart health [6], vitamins that significantly impact sexual health and fertility [7], phenolic compounds-that have a role in antioxidant and anti-inflammatory [8]. Quercetin is the most effective antioxidant that' is found in *P. guajava* [9]. *P. guajava* leaves are primarily used in traditional medicine for the management of gastrointestinal ailments such as diarrhea, stomachache, gastroenteritis, indigestion, and dysentery, as well as cosmetic issues including skin infections, skin aging, and ulcers [10]. *Aspergillus terreus* has a diverse range of applications, including: being used to manage diseases caused by *Rhizoctonia solani* in *Vicia faba* [11], as well as early blight in eggplant [12]. *A. terreus* exhibits antifungal activity against fungi responsible for mucormycosis [13]. *A. terreus* is considered an initial source for the cholesterol-lowering drug lovastatin (mevinolin) [14]. *A. terreus* is used to produce important organic acids like itaconic acid and cis-aconitic acid, as well as enzymes like xylenase [15].

The main objective of this study is to isolate and identify endophytic fungi from the *P. guajava* plant with the capability to inhibit microbial growth. These fungi could serve as promising, safe, and effective alternatives to traditional antibiotics in combating pathogenic bacteria and fungi, thereby helping to curb the rise of antibiotic-resistant strains. This research aimed to provide scientific justification for the antibacterial efficacy of the ethyl acetate extract (EtOAcE) *of A. terreus* against a diverse array of microorganisms. Furthermore, an assessment was conducted to determine the appropriate therapy dosages and potential clinical manifestations caused by *A. terreus* EtOAcE, with a focus on acute oral toxicity. In addition to provide a scientific rationale for the antibacterial effectiveness of leafves extract obtained from a medicinal plant toward a wide variety of microorganisms. Furthermore, the primary objective of this research was to evaluate the acute oral toxicity of the *A. terreus* extract with the purpose of determining the ideal therapeutic dosages for ensuring its safety for humans, especially for the investigation and creation of novel medications.

2. Materials and methods

2.1. Source of plant sample

In this study, healthy *P. guajava* leaves were collected from kfor Bolshay, Kafr Al-Zayyat City, Gharbia Government, Egypt (30°51′41.4″N 30°47′46.3″E) and transported to the laboratory in an ice box in March 2023, stored in sterile polyethylene bags at 4°C.

2.2. Isolation of endophytic fungi

Leaf segments of 1 cm^2 from *P. guajava* plants were cut, rinsed with tap water, and then sterilized for 60 s with 70% ethanol (CH_3CH_2OH) and 60 s with 4% sodium hypochlorite (NaOCl). The sterilized leaf segments were plated on potato dextrose agar medium (PDA) (Oxoid) supplemented with chloramphenicol (three replicates). The plates were incubated at 27 ± 2°C for three weeks and checked daily. Hyphal tips from the cultivated leaf segments were sub cultured onto fresh, sterilized PDA media [16].

2.3. Identification of endophytic fungus through phenotypic and genotypic investigations

A fungal isolate was morphologically identified in accordance with previous research [11,12,17]. The morphological characteristics of the fungus, such as its pigment, surface, and colony diameter, were noted along with its vegetative and reproductive structures. According to Khalil et al. [18], small subunit ribosomal RNA (18S) was used for the molecular identification of fungal endophytes. After making the necessary manual adjustments, codons aligned the acquired ITS sequences. The resulting sequence was compared to similar sequences from DNA databases using the National Center for Biotechnology Information's (NCBI) BLAST search program. Molecular Evolutionary Genetics Analysis software MEGA-X was used to perform phylogenetic trees.

2.4. Fungal metabolites extraction

For 21 d, an endophytic fungus was grown in a PDB medium (Oxoid) at 27 ± 2°C in a static environment. The fermentation broth was filtered under aseptic settings and then extracted by ethyl acetate ($CH_3COOC_2H_5$) (1:1) and the mixture was vortexes for 10 min, after which it settled for 5 min to produce two distinct, transparent layers. The separating funnel was then used to separate the organic layer ($CH_3COOC_2H_5$) from the aqueous layer. The collected organic phase was evaporated using a rotary evaporator at 45°C [13].

2.5. GC–MS analysis

Gas Chromatography Mass Spectrometry analysis was carried out using a capillary column (length 30 m, thickness 0.25 μm, internal diameter 0.25 mm) on a Thermo Scientific Trace GC1310-ISQ mass spectrometer (Austin, Texas, USA). One μL fungal

extract was injected at 250°C using helium as the carrier gas with a split ratio of 1:30. The oven temperature started at 50°C and was maintained for 5 min and then ramped up to 230°C at a rate of 5°C per minute. The mass spectrometer operated in electron ionization (EI) mode at 200°C and 70 eV with a scan range of 40 to 1000 *m/z*. The observed compounds compared with those of the identified compounds were cataloged in WILEY 09 (Wiley, New York, NY, USA).

2.6. Microorganisms

Four typical varieties of bacteria *Escherichia coli* (MTCC 443), *Enterobacter aerogenes* (MTCC 111), *Bacillus sphaericus* (MTCC 511) and *Staphylococcus aureus* (MTCC 87) were used to assess the antibacterial properties of the EtOAcE in the study.

2.7. Determination of antimicrobial activity by Kirby–Bauer disk diffusion method

The Agar-well diffusion method was applied for the determination of antibacterial properties [19,20]. Firstly, several tested colonies of bacteria were inoculated in the brain–heart infusion broth and incubated at 37 ± 2°C until they reached the turbidity of 0.5 McFarland standard (approximately = 1×10^6 CFU/mL). After solidification, (100 μL) of the antimicrobial agent or fungal EtOAcE solution at the desired concentration (100 mg/mL, 200 mg/mL and 400 mg/mL) was introduced according to Kirby–Bauer disk diffusion method [21]. The inhibition zones measurement was done in triplicate for each sample. Fungal extracts were manipulated to determine their minimum inhibitory concentration (MIC) using the agar dilution method by preparing different concentrations of extracts (from 600 mg/mL to 4.6 mg/mL) through two-fold serial dilution. For 18 to 24 h, all concentrations of the fungal extract that show detectable activity are subcultured onto the suitable medium plate at 37°C. Next, on the newly infected agar plates, the quantity of fungal extract that did not show any bacterial development was determined to be the smallest bactericidal concentration, or MBC. Every test was run in duplicate [22].

2.8. Acute oral toxicity: Experimental procedure

An acute toxicity study was conducted in accordance with the test Guidelines 425 (Acute Oral Toxicity–Fixed dosage technique) established by the Organization of Economic Cooperation and Development (OECD), with minor adjustments. Twenty adult male albino mice that were carried out in the study [23]. The experimental twenty male mice were randomly chosen ranginged from 8- to 12-week-old mice, labeled to enable individual identification, and housed in their cages for 3 d prior to dosing to facilitate adaptation to the laboratory environment. Animals used in experiments included rats that were obtained from an Egyptian holding firm for biological goods and vaccines (VACSERA). In the animal house under the authority from Zoology Department, Faculty of Science, mice were acclimatized before the start of the experiment at standard laboratory settings (12 light/12 darkness (h), room temperature at 25°C, tap water and free access to typical commercial rodents' food ad libitum. Then, the animals were measured in order to determine their body weight then were transferred to metallic cages for grouping. The animals exhibited a body weight ranging from 20 to 23 g at the beginning of their dosage [24].

2.9. Experimental design

A total of twenty male albino mice were included in the current investigation. The animals were randomized to control and various treatment groups in a random manner. They were then split into four groups, with each group consisting of five mice; Group 1 mice served as controls and were administered distilled water for a vehicle while animals in groups 2, 3, and 4 were given *A. terreus* EtOAc*E* by intragastric gavage every day for 14 d at dosages of 20, 40 and 80 mg/kg bw, respectively. The animals were monitored for signs of toxicity every day during this time, and for a total of 14 d, their water and food intake as well as death rates were noted every other day. However, animals were allowed to stand for one week in order to adjust before commencing the experiment. Animals were postponed from receiving the following dosage until the survival of the animals that had been dosed before was assured.

2.9.1. Subacute toxicity

The determination of the crude *A. terreus* EtOAcE was based on the body weight of the mice and dissolved, considering the supplied amount, which is limited to 1 mL per kilogram of body weight. Next, oral gavage was used to administer the diluted extract to the treatment groups and the solvent alone to the control groups. After the administration, each mouse was carefully observed for the first 30 min, then every 2 h for the next 2 h, every 6 h for the next 42 h, and every day for the next 14 d. Systematic documentation was conducted to record several findings, including alterations in food and water consumption, respiratory patterns, occurrences of diarrhea, behavioral tendencies, and death rates. Additionally, the body weight was assessed at the beginning, as well as after 7 and 14 d of therapy.

2.9.2. Blood sample collection

On day 14 of the study, the mice had a 12-h fasting period, were weighed, and had their blood samples collected via isoflurane inhalation. In order to collect the blood samples, blood capillary tubes from each mouse were used to puncture the retro-orbital venous plexus. Blood samples were collected on a tube with anticoagulant (EDTA) for the goal of conducting a hematological analysis.

2.9.3. Hematological assay

Blood samples were collected on EDTA to assess several hematological parameters using a hematological cell counter (Beckman Coulter DxH 500), including Erythrocytes/RBCs, Erythrocyte Indices, Leucocytes-WBCs, differential leucocytes, and platelet count.

2.9.4. Data analysis and interpretation

The represented data was carefully examined to ensure they were complete and consistent. Subsequently, data were subjected to analysis using a one-way analysis of variance (ANOVA) by SPSS version 26 for Windows, accompanied by the least significant difference (LSD) test to investigate significant differences in the antibacterial efficacy of crude *A. terreus* EtOAcE against different strains of bacteria and their effect on the body weight of the mice. The experimental data is expressed by the mean in conjunction with the standard error. The finding was determined to be of statistical significance at a significant level of $p < 0.05$ [24].

3. Results and discussion

3.1. Isolation and identification of endophytic fungi

The fungal isolate, obtained from surface sterilized *P. guajava* leaves parts, was morphologically identified as *A. terreus*. Macroscopic identification revealed granular conidial formation at 25°C on PDA, with colonies developing modestly up to 60 mm in diameter after one week. Colonies exhibit a buff to yellow surface and Cleistothecia are present, with the reverse showing a hyaline orange to deep center. Surrounding Hull cells are either buff or dull

yellow in color. Microscopic examination shows septated mycelium, biseriate conidial heads, and smooth-walled conidiophores up to 300 μm in length. Pyriform vesicles are smooth and globose, measuring tiny (2–2.5 μm) with a width of up to 12 μm including conidia (Fig. 1a, b, c). Molecular identification revealed that the isolated fungal strain is like *A. terreus* OM250078.1 with 98.56% identity, *A. terreus* JN831364.1 with 98.64%, *A. terreus* MW8814252.1 with 98.71%, and *A. terreus* GU227345.1 with 98.76%. It is recorded in the gene bank as *A. terreus* (OR125572). Phylogenetic analysis of the fungal strains revealed a 96% identity with related species using BLAST programs (Fig. 1d). In numerous earlier investigations, *Aspergillus* species have been identified as endophytic fungi isolated from inside healthy plants [25,26]. *Aspergillus* is considered one of the most widespread fungi within plants [13,27]. Endophytic fungi often establish commensal or mutualistic relationships with the plants they inhabit. *Aspergillus,* known for its versatility, can colonize a variety of plant species, making it a common endophytic fungus. *Aspergillus* can form endophytic associations with various plant parts including leaves, stems, roots, and seeds. *Aspergillus* endophytes provide a variety of advantages for plants, including the ability to produce compounds like phytohormones, which promote plant growth and development. These endophytes can also enhance plant resilience to various challenges such as infections, salt stress, and drought [26]. They achieve this by either activating the plant's defense mechanisms or producing secondary metabolites with antibacterial properties. Furthermore, *Aspergillus* endophytes aid in the breakdown of organic matter and the release of nutrients into the soil, contributing to the nutrient cycle. By solubilizing minerals and increasing nutrient availability, they can also enhance the plant's efficiency in absorbing nutrients [25]. *A. terreus* possesses characteristics that contribute to its potential as an endophyte. It can produce a variety of secondary metabolites, including enzymes and bioactive compounds, which may promote plant growth or defend against pathogens [12].

3.2. GC–MS analysis

A typical spectral output for each chemical present in the examined samples is provided by GC-MS analysis. Thus, GC-MS has gained widespread recognition as a key technological platform for characterizing secondary metabolites in recent years. The GC-MS chromatograms of *A. terreus* EtOAcE revealed the presence of 32 different chemicals, as detailed in Table 1. 1,2-Benzenedicarboxylic acid, diisooctyl ester (37.66%), Hexyl oxecan-2-one (16.35%), Phenol, 3,5-dimethoxy-, acetate (7.65%), 2-Propenoic acid, butyl ester (7.5%), 9-Octadecenamide, (Z) (3.61%), and 2,2′-methylenebis(6-(1,1-dimethylethyl)) (2.66%) are the main components of the fungal extract. These are followed by Hexadecanoic acid, methyl ester (1.79%), 10-Octadecenoic acid, methyl ester (1.79%), P-xylene (1.65%), 4-Methyl-2-pentanol (2.66%), and Hex-3-enyl isobutyl ester (1.97%). The components of fungal extracts exhibit various biological activities, including hepatic-protective (Octadecenoic acid methyl ester and Spirol (1,3-dioxolane)-2,3′-(5′-androsten-16′-ol)), Hypocholesterolemic (Octadecenoic acid methyl ester), anti-inflammatory (Docosene), anticancer (Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, Podocarpa-8,11,13-triene-12,13-diol, cis-11-eicosenoic acid, and Hexadecanoic acid 2-hydroxy-1,3-propanediyl ester), antitumor (Docosene), and antihistaminic (Octadecenoic acid methyl ester). Some components have antioxidant activity, including Propenoic acid butyl ester, Cyclononasiloxane octadecamethyl, Hexadecanoic acid, Docosene, Tetracosene, Oleic acid eicosyl ester, Phenol

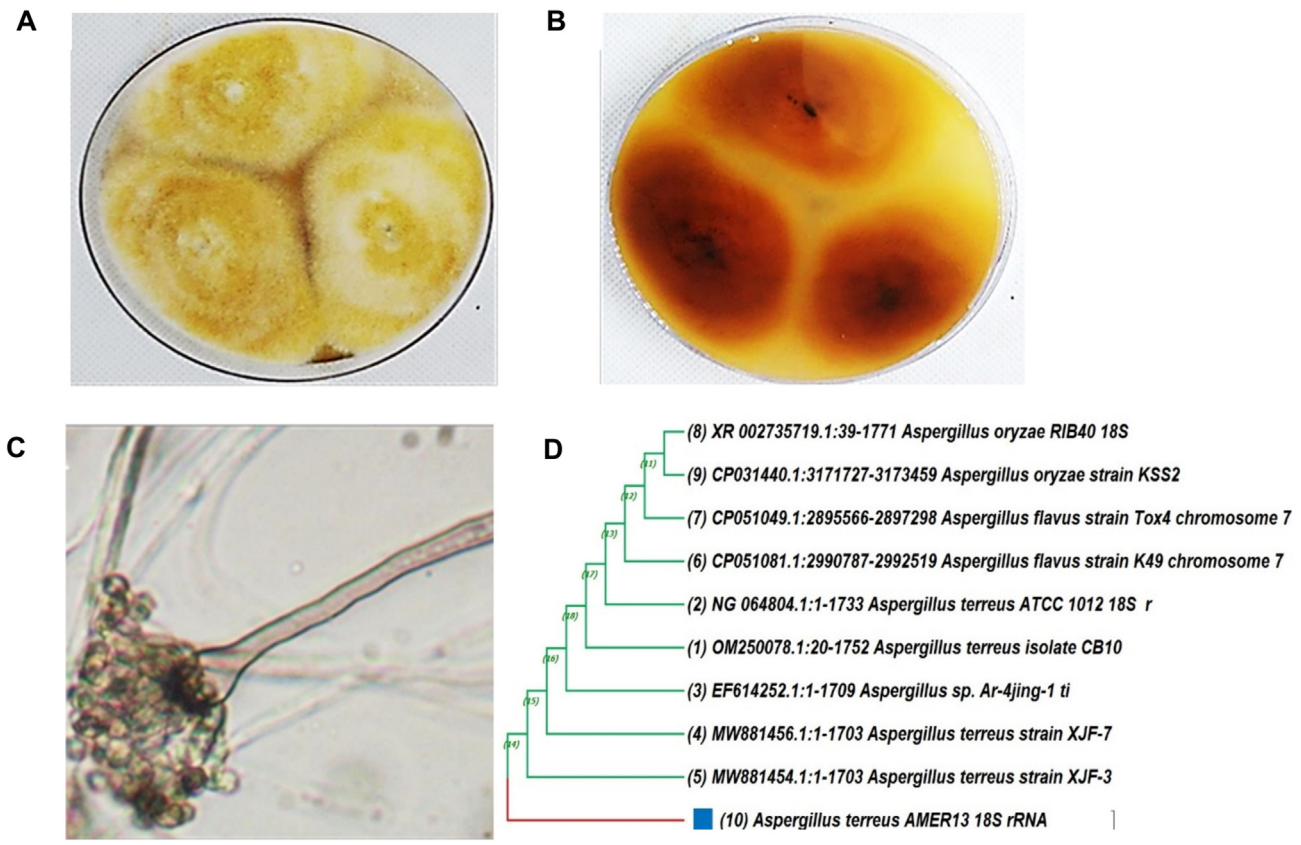

Fig. 1. Morphological and Phylogenetic tree of genetically identified *A. terreus* (OR125572). (A) surface of culture on PDA; (B) reverse color of culture on PDA; (C) conidiophore, head and conidia under the light microscope (400X); (D) Phylogenetic tree.

Table 1
Compounds Identified by GC-MS in *A. terreus* EtOAcE.

No.	Compounds	RT (min)	Peak area %	Activity	References
1	*P*-Xylene	6.21	1.65	Antimicrobial	[16]
2	2-Propenoic acid, butyl ester	6.93	7.5	Antimicrobial and Antioxidant	[41]
3	1-Octadecene	43.59	0.48	Antifungal	[42]
4	2-Hexadecanol	43.64	0.13	Antimicrobial and Anti-nematodial	[43]
5	Hexyl-oxecan-2-one	44.47	16.35	Choline esterase inhibitor	[44]
6	Actinomycin C2	45.24	0.08	Antimicrobial	[45]
7	Ethyl-octahydro-inden-3a-ol	45.63	0.16	Antifungal	
8	Cyclononasiloxane, octa-deca-methyl	46.03	0.06	Antimicrobial and Antioxidant	[46]
9	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	46.12	0.22	Anticancer	[47]
10	Podocarpa-8,11,13-triene-12,13-diol	46.64	0.19	Anticancer	[48]
11	2,5-Cyclohexadiene-1,4-dione, 2,6-bis (1,1-dimethylethyl)	46.78	0.11	Antifungal	[49]
12	Hexadecenoic acid	48.77	0.25	Antioxidant and pesticide	[50]
13	2,5-Di-tert-Butyl-1,4-benzoquinone	48.94	0.26	Anticandidal, antibacterial	[51]
14	Isochiapin B	49.56	0.75	Antidiabetic	[52]
15	1-Docosene	50.13	0.83	Anti-inflammatory, Antioxidant and Anti-tumor	[53]
16	Spiro(1,3-dioxolane)-2,3′-(5′-androsten-16′-ol)	50.31	0.76	Hepatoprotective	[54]
17	cis-11-Eicosenoic acid	50.35	0.12	Anticancer	[55,56]
18	10-Octadecenoic acid, methyl ester	52.79	1.79	Hepatoprotective, Antihistamine, Hypocholesterolemic, Anti-eczemic	[57]
19	Oleic acid, 3-(octadecyloxy) propyl ester	54.03	0.25	Antifungal	[58]
20	Tetracosene	56.69	0.75	Antimicrobial and antioxidant	[59]
21	Spermine	56.87	0.81	Antioxidant	[60]
22	Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester	59.22	1.83	Anticancer	[61]
23	Oleic acid, eicosyl ester	61.79	0.12	Antibacterial and antioxidant	[62]
24	2,2′-methylenebis[6-(1,1-dimethylethyl)-4-methyl	62.26	2.66	Antibacterial	[63]
25	Phenol, 3,5-dimethoxy-, acetate	63.93	7.65	Antimicrobial and antioxidant	[64]
26	Trilinolein	64.43	0.41	Antioxidant	[65]
27	Glycidyl palmitoleate	64.68	0.61	Antimicrobial	[66]
28	Heptanoic acid, docosyl ester	65.21	0.28	Antimicrobial	[67]
29	1-Hexacosene	69.03	0.33	Antibacterial and antioxidant	[68]
30	9-Octadecenamide, (Z)-	72.42	3.61	Antibacterial	[69]
31	Supraene	74.34	0.78	Antioxidant	[70]
32	β-Sitosterol	84.15	0.55	Antioxidant	[71]

3,5-dimethoxy acetate, Trilinolein, hexacosene, supraene, and β-sitosterol. Some components have antimicrobial properties, including Phenol, 3,5-dimethoxy- acetate; Propenoic acid, butyl ester; Hexadecanol; Actinomycin C2; Cyclononasiloxane, Octadecamethyl; Tetracosene; Glycidyl palmitoleate; and Heptanoic acid, docosyl ester. Some components have fungicidal [28,29] (Octadecene, Ethyl-octahydro-inden-3a-ol, cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl), oleic acid, 3-(octadecyloxy)propyl ester), Anticandidal (2,5-di-tert-butyl-1,4-benzoquinone), antinematedal (Hexadecanol), enzyme inhibitory (Hexyl oxecan-2-one, Octadecenoic acid, methyl ester), insecticidal [30] (Hexadecanoic acid), ant-diabetic (Isochiapin B), and anti-eczema (Octadecenoic acid, methyl ester) properties.

Numerous studies have reported that bioactive substances with antibacterial, antioxidant, anticancer, and antitumor properties have been isolated from *P. guajava* leaf extract [31]. Hepatic-protection, endothelial progenitor cells, anti-inflammatory, antispasmodic, anti-inflammatory, antibacterial, anti-hyperglycemic, analgesic, and anti-diarrhea properties [32]. Abundant chemically active compounds can be produced by endophytic microorganisms, as demonstrated by earlier research. Consequently, endophytes are a reservoir of an extensive collection of biologically active compounds that offer advantages to both plants and humans [2,11,33]. Previous studies have demonstrated the ability of fungi isolated from *P. guajava* to produce antioxidants and bioactive compounds with antifungal and antibacterial properties that are harmful to humans as *Candida albicans* [34,35]. AT is recognized for its ability to synthesize lovastatin, a drug used to reduce cholesterol levels in humans. In addition to lovastatin, AT produces several bioactive substances including Sulochrin, Terretonins, Asterriquinones, and Butyrolactones [36,37]. Finally, the phytochemical compounds found in the leaves of *P. guajava* and its endophytic fungal isolate are correlated through complex metabolic pathways and interactions. The endophytic fungal isolate influences the production of bioactive compounds by the plant, and the fungus itself produces compounds that enhance the bioactivity of the plant's compounds [38,39,40].

3.3. Antimicrobial activity

The average Zone of suppression created by *A. terreus* EtOAcE evaluated against *S. aureus* (MTCC 511), *B. sphaericus* (MTCC 511), *E. aerogenes* (MTCC 111), and *E. coli* (MTCC 443). *B. sphaericus* exhibited the biggest average zone of inhibition; the inhibition zone against this bacterium varied from 18 mm by 100 mg/mL fungal extract to 38 mm by 400 mg/mL ethyl acetate extract. At all matching dose, Table 2 and Fig. 2 demonstrated a smaller inhibitory zone against *E. coli* than the other investigated microorganisms at 100 mg/mL which had the lowest limitation radius, measuring 18 mm. The effect of ciprofloxacin (15 μg) on all tested bacterium revealed more inhibition of all treatment and doses, with a significant difference at $p < 0.05$. The potency of ethyl acetate extract on *B. sphaericus* was better than other tested bacteria. To determine the MIC value, each tested species was initially examined at an elevated level of 600 mg/mL until being serially diluted to 0.64 mg/mL. According to the investigation, the MIC value of the ethyl extract was consistent with its preliminary antimicrobial activity screening against the majority of the bacteria. The ethyl acetate extract suppressed *E. aerogenes* at 18.75 mg/mL which have a lower MIC while *S. aureus* has the

Table 2
Measurement of the inhibition zone diameter to using the Kirby–Bauer disk diffusion method.

	Inhibition Zone Diameter (mm), including hole diameter (7 mm)				
	Conc. (mg/mL)	*E. coli*	*E. aerogenes*	*B. sphaericus*	*S. aureus*
Ethyl acetate extract	100	18 ± 0.9	22 ± 2.1	26 ± 0.9	21 ± 0.9
	200	24 ± 1.1	29 ± 1.7	31 ± 0.6	29 ± 1.8
	400	29 ± 1.3	33 ± 0.5	38 ± 1.2	31 ± 1.2
Distilled water	–	7 ± 0.0	7 ± 0.0	7 ± 0.0	7 ± 0.0
Vancomycin	30 µg	31 ± 1.4	32 ± 0.9	34 ± 0.6	30 ± 0.7
5% tween 80	–	7 ± 0.0	7 ± 0.0	7 ± 0.0	7 ± 0.0

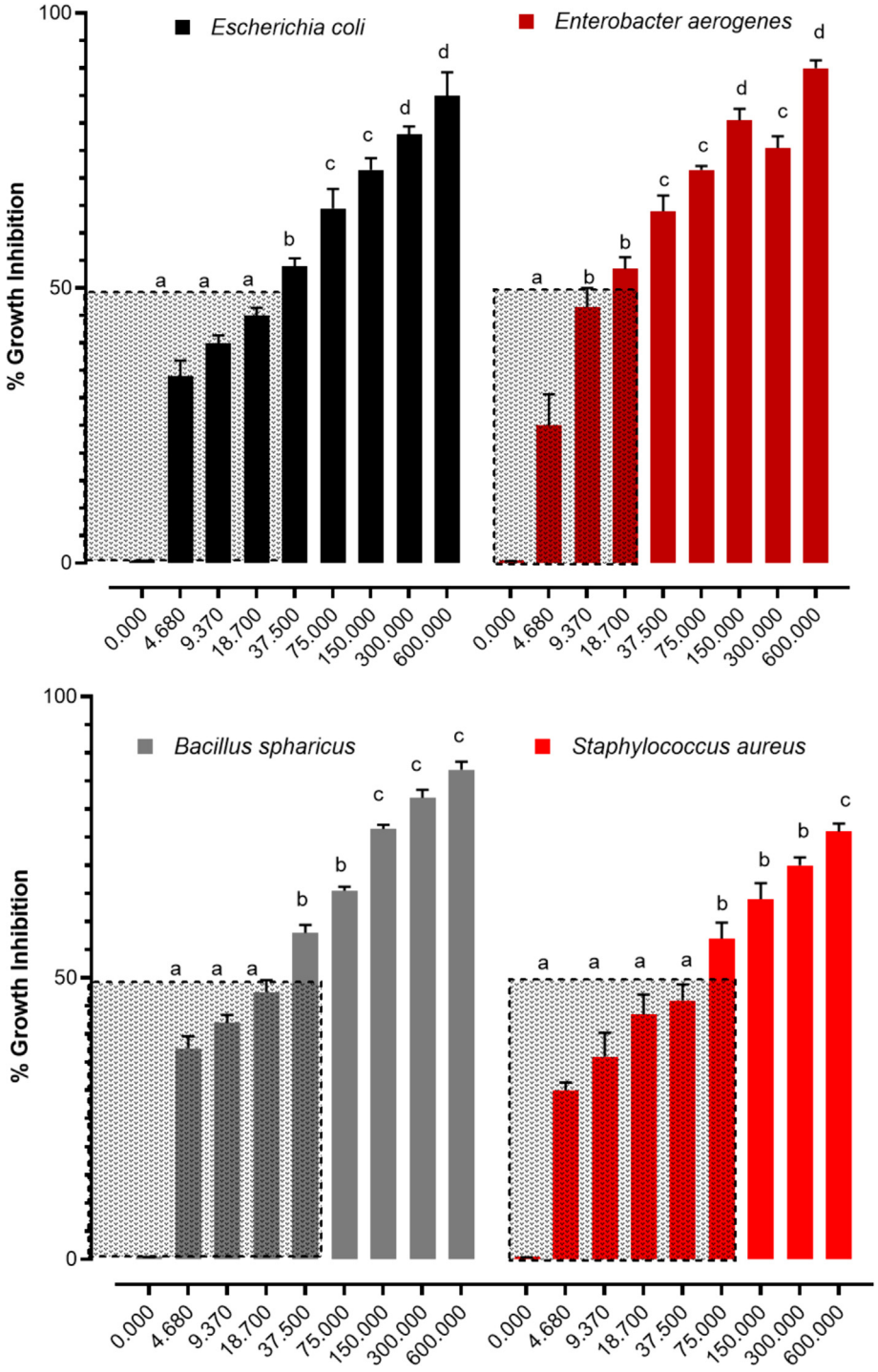

Fig. 2. Effect of different concentration fungal EtOAcE on the growth of tested strain; Growth analysis curves measured by monitoring the optical density (OD) at 600 nm after 24 h of incubation at 37°C. (a,b,c and d revealed significance letters).

highest MIC value of 75.0 ± 0.0 mg/mL compared to other examined bacteria *E. coli* and *B. sphaericus* have MIC values at 37.5 mg/mL. These might be the reasons of the two species have lower MIC and MBC values compared bacteria with longer boundaries of inhibition. Thus, among the extracts, those containing extracts made with ethyl acetate may have a greater capacity to penetrate the outer membrane of gram-negative bacteria and disrupt cellular function, metabolism, and loss of cellular contents, resulting in suppression and mortality of the bacteria. Gram-positive bacteria were more responsive to the extracts than Gram-negative bacteria. Many additional researches on additional medicinal plants have found that gram-positive bacteria are more susceptible to the antibacterial activities of plant extracts than gram-negative bacteria [72]. This might be because gram-negative bacteria have an outer layer made of a high level of lipopolysaccharides, which operate as an obstacle to numerous contaminants in the environment, particularly medications [73]. MBC for *E. coli*, *E. aerogenes* and *B. sphaericus* were observed at 75 mg/mL respectively while *S. aureus* was 150 mg/mL.

3.4. Acute oral toxicity study

3.4.1. Behavioral assay and LD_{50}

The results of an acute oral toxicity study showed that the *A. terreus* extract appeared to be safe up to the dose of 1 mL/kg. Test parameters of Breathing, urination, food, and water intake were assessed in Table 3 and Table 4 as well as the series of Fig. 3. Urination, diarrhea, drowsiness, sedation, food consumption and water intake as well as mortality Rrate were observed at administrated doses of 0.25, 0.5 and 1.0 mL /kg, respectively. However, no signs of toxicity were observed in any other groups. The research generally showed that most of the parameters were not harmful;, however, 20% of the mice in the groups given *A. terreus* extract at dosages of 0.5 and 1.0 mL /kg, respectively, died before the 14th d. Therefore, the LD_{50} of the extract might be considered to be greater than 1 mL /kg administration of *A. terreus* extract while there is no sign of toxicity in at 7 d of treatment. The study was designed to mitigate consumer encounters with potential risks linked to the consumption of *A. terreus*. In the context of acute oral toxicity, it was noted that mice at doses of 40 and 80 mg/kg body weight did not exhibit any abnormalities. However, death was seen after 14 d. Consequently, it can be concluded that the LD_{50} of the *A. terreus* extract exceeds 160 mg/kg. Consequently, the extract is reasonably harmless since compounds with an LD_{50} ranging from 40 mg/kg to 80 mg/kg orally are regarded to induce low toxicity; however, under some circumstances, they may pose a risk to susceptible populations [23]. The results are in the same way as that conducted by the study of Sekhar et al. [74] The study demonstrated that the aqueous bark extract of *A. terreus* exhibited an LD_{50} value over 5000 mg/kg, and that the animals maintained their health status during an additional 10-d observation period. Nevertheless, another study revealed that the *P. guajava* leaf extract at a dosage of 2000 mg/kg had an LD_{50} higher than the study of Roy et al. [75].

3.4.2. Body weight

During the initial day as well as the 7 and 14 d, the body weights of every experimental group were measured as established in Fig. 3 and Fig. 4. At these days, in all treated groups administrated 3 different doses of *A. terreus* extract; in comparison to the negative control group administered Dis. Water, there are no statistically significant changes ($p < 0.05$) in body weight while the percent of change was elevated to 42.9% in group administrated of *A. terreus* extract at dose of 0.5 mL/kg in compared to 34.8% and 40.9% at the doses of 0.25 and 1.0 mL/kg, respectively. Reduction of body weight by more than 10% did not occur in our study. Nevertheless, the absence of weight loss in the current study indicates that, after 14 d of dosing, the plant was not hazardous. Our results

Table 3
General observation and mortality rate after administration of serial dilution of *A. terreus* EtOAcE in control and treated groups after 7 and 14 d

Test Parameters	General observation						
	Control	7 d					
	Dis. water	0.25 mL/kg	0.5 mL/kg	1.0 mL/kg	0.25 mL/kg	0.5 mL/kg	1.0 mL/kg
Food intake	Normal	Normal	Normal	Normal	Normal	Abnormal	Abnormal
Water intake	Normal	Normal	Normal	Normal	Abnormal	Abnormal	Abnormal
Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent	Present
Urination	Normal	Normal	Normal	Normal	Normal	Abnormal	Abnormal
Breathing	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Skin color	Normal	Normal	Normal	Normal	Normal	Normal	Abnormal
Drowsiness	Absent	Absent	Absent	Absent	Absent	Present	Present
Sedation	Absent	Absent	Absent	Absent	Present	Present	Present
Mortality Rate	All Alive	0%	0%	0%	0%	20%	20%

Mean value represents mean of 5 records ± SE while the serial dilution is being used at all extracts.

Table 4
Chang in Body Weight (g) for control and treated groups administrated *A. terreus* crude extract after 7 and 14 d of treatments.

	% of body weight (g) change				
Dose	**Dis. water**	**1st dose (0.25 mL)**	**2nd dose (0.5 mL)**	**3rd dose (1.0 mL)**	**F-value**
Time	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	
Initial day	22.0 ± 1.2	23.0 ± 1.2	21.0 ± 1.2	22.0 ± 1.2	0.50
7th d	26.0 ± 1.2	27.0 ± 1.2	27.0 ± 1.2	26.0 ± 1.2	0.25
% of change	18.2%	17.4%	28.6%	18.2%	
14th d	34.0 ± 1.2	31.0 ± 1.2	30.0 ± 1.7	31.0 ± 1.2	1.71
% of change	54.5%	34.8%	42.9%	40.9%	

Mean value represents mean of 5 records ± SE. while serial dilution is being used at all extracts. Percent of change are calculated from the initial body weight of mice per groups.

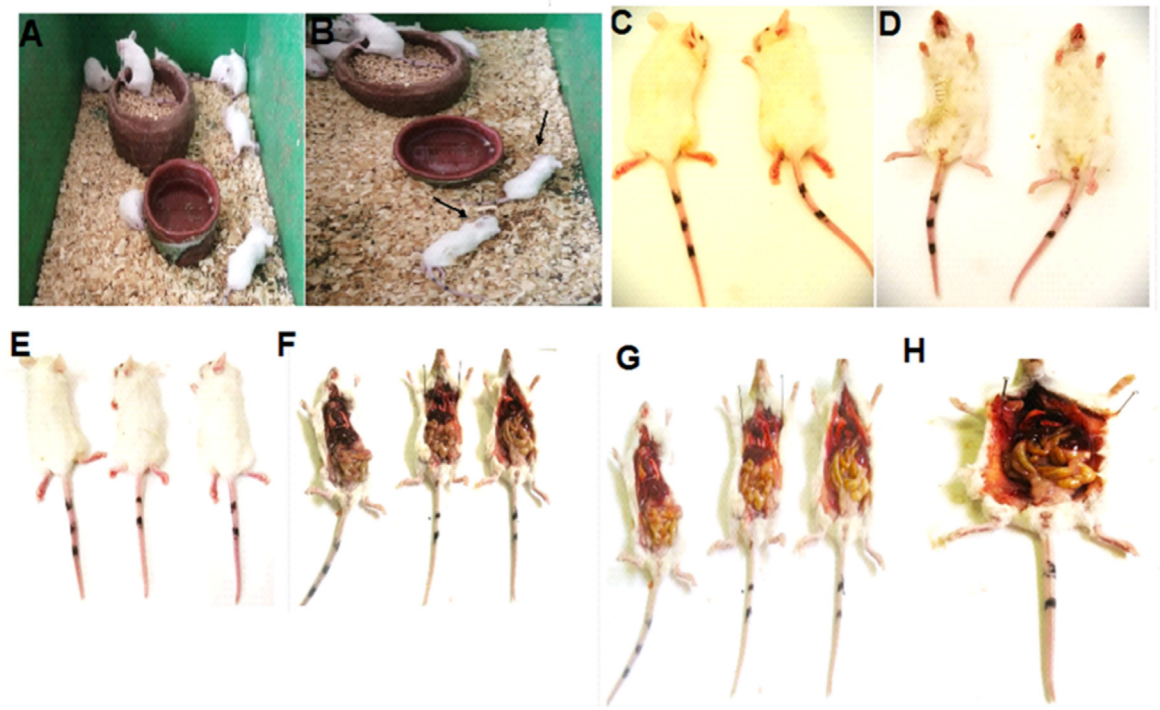

Fig. 3. Illustrated normal mice After 7 d of treatment while there is no sign of morbidity as well as morbidity (A); Illustrated a sign of toxicity including increase water intake rather than food intake as well as morbidity and mortality are occurred after 14 d of treatment (B); Represented a sign of toxicity including drowsiness at doses of 40 and 80 mg respectively per kg after 14 d of treatment (C); Represented a sign of toxicity including urination and abnormal skin color at doses of 40 and 80 mg respectively per kg after 14 d of treatment (D); Represented a sign of toxicity including injury at the viscera, malformation of internal organs which induced mortality at doses of 40 and 80 mg respectively per kg after 14 d of treatment (E,F,G,H).

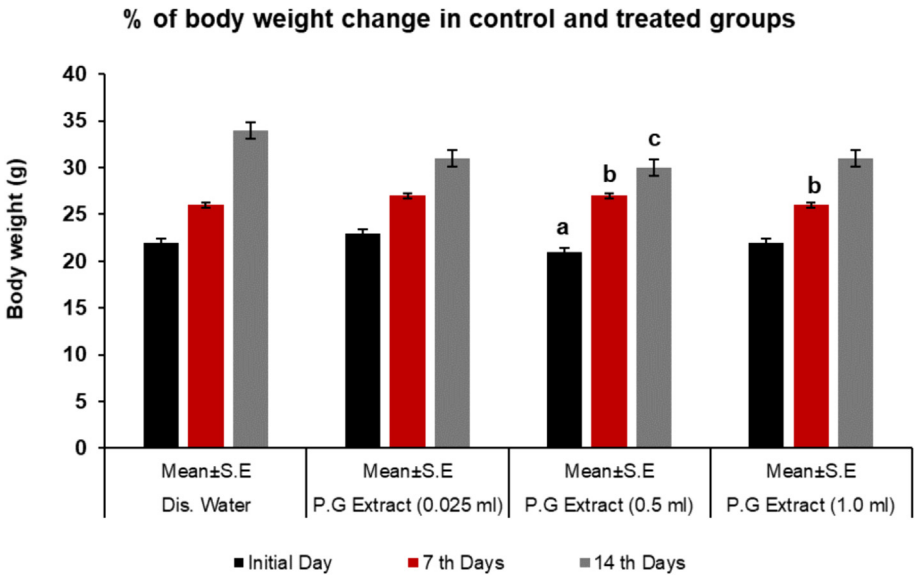

Fig. 4. The effect of the *A. terreus* extracts on the body weight changes at different times of treatment. Transcript letter a represented the comparison of body weight at the baseline, while the transcript letter b represented the comparison between all experimental groups after 7 d of treatment while the same transcript letter explained that no significant differences. Furthermore, the transcript letter c represented the comparison between all experimental groups after 14 d of treatment.

conflict with a research that found that ant nutritional compounds like the tannins and saponins in this plant lead the body to absorb nutrients less efficiently [76].

3.4.3. Haematological study in vivo

The Hematological parameters were represented in Table 5 and Table 6 after. One-way ANOVA for comparison of treated groups with the control group. According to hemoglobin level which is related to RBCs, Haematocrite (Hct) and Erythrocytes indices (MCH and MCHC), there are no significant changes ($p < 0.05$) occurred in all subject groups. Furthermore, there are no significant changes in Platelets Count find in comparison between all subject groups. However, the concentration of MCV presented a significant decrease ($p < 0.05$) in all treated groups administrated

Table 5
Mean ± SE values of Hb %, RBCs, Hct and Erythrocytes indices (MCV, MCH and MCHC) as well as Platelets count in all subject groups after 7 and 14 d of treatments.

Parameters	HGB (g/dl)	RBCs (10^6 cell/mm^3)	Erythrocytes indices				Platelets (10^3 cell/mm^3)
			Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	
Groups	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Dis. Water	13.4 ± 1.4	5.3 ± 0.1	40.1 ± 4.3	75.8 ± 8.9	25.3 ± 3.0	33.3 ± 0.0	595.0 ± 111.8
1st dose (0.025 mL)	13.6 ± 1.3	7.4 ± 0.9	38.4 ± 4.3	52.1 ± 0.5	18.6 ± 0.6	35.7 ± 0.8	444.3 ± 82.5
2nd dose (0.5 mL)	11.4 ± 0.8	7.6 ± 1.6	41.2 ± 8.3	54.4 ± 0.8	16.2 ± 3.1	29.8 ± 5.7	844.7 ± 182.6
3rd dose (1.0 mL)	12.8 ± 0.8	6.7 ± 0.6	35.8 ± 2.9	53.6 ± 1.3	19.2 ± 0.6	35.8 ± 0.8	502.7 ± 139.5
p – value	NS	NS	NS	$p \leq 0.05$	NS	NS	NS
F-value	0.84	1.20	0.19	6.2	3.1	0.95	1.73

Mean value represents mean of 5 records ± SE. NS represented that there is no significant difference at p-value <0.05.

Table 6
Mean ± SE values of WBC.s and Differential leucocytes (Lymphocytes, Monocytes, Neutrophils, Eosinophiles and Basophils) in all subject groups after 7 and 14 d of treatments.

Parameters	Leucocytes count and it is differential					
	WBC.s (10^3 cell/mm^3)	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)	Eosinophils (%)	Basophils (%)
Groups	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Dis. Water	6.0 ± 0.4	83.0 ± 2.9	5.3 ± 0.3	9.0 ± 2.9	2.3 ± 0.3	0.3 ± 0.3
1st dose (0.025 mL)	6.2 ± 1.3	65.6 ± 9.9	21.5 ± 2.0	10.3 ± 3.5	0.1 ± 0.1	2.2 ± 0.3
2nd dose (0.5 mL)	5.5 ± 1.1	65.4 ± 6.2	15.6 ± 3.6	14.8 ± 2.4	1.3 ± 1.0	3.0 ± 0.5
3rd dose (1.0 mL)	5.9 ± 1.0	66.1 ± 9.6	17.4 ± 4.7	13.2 ± 4.3	1.2 ± 1.0	2.1 ± 0.3
p – value	NS	NS	$p \leq 0.05$	NS	NS	$p \leq 0.01$
F – value	0.09	1.25	4.8	0.62	1.5	10.1

Mean value represents mean of 5 records ± SE. NS represented that there is no significant difference at p-value <0.05.

A. terreus extract at the different doses (20, 40 and 80 mg/kg b.w) respectively when compared to negative control group which suggested that orally administration to *A. terreus* extract may be caused microcytic anemia due to iron deficiency or poisoning related to administration. The oral administration of *A. terreus* may lead to microcytic anemia as a result of iron shortage or toxicity occurring during administration. Hemoglobin, hematocrit, MCH, and MCHC levels were not significantly different from the control group during this study, indicating that the blood's ability to carry oxygen to the tissues has not been compromised. Consequently, the hematopoietic system has not been affected [77]. On the other hand, the percentage of Monocytes showed a significant increase rather than leukocytes in all treated groups administrated *A. terreus* extract at the different doses (20, 40 and 80 mg/kg b.w) respectively when compared to negative control group while A high monocytosis can indicate an infection or immunomodulatory responses related to administration of *A. terreus* extract. However, in comparison to the negative control group, there are no significant differences ($p < 0.05$) in the leucocyte count or its differential components (lymphocyte, eosinophil, neutrophil, and basophil) between the treatment groups. In contrast, the experimental animal strain used in the study of Uboh et al. [78] reported that the aqueous *P. guajava* leaves extract, when given to male rats for 30 d, had hematopoietic potential, as evidenced by the increased number of leucocytes reported in this study at 1000 mg/kg body weight.

4. Conclusions

From our findings, we can conclude that the endophytic fungus, *A. terreus* (OR125572), was phenotypically and genetically identified from *P. guajava* plant leaves. This endophytic fungus has been shown to be a potent source of biologically active organic chemicals. Ethyl acetate extract suppressed *E. aerogenes* which have lower MIC while *S. aureus* has the highest MIC value compared to other examined bacteria. Based on the findings of an acute oral toxicity study, it can be concluded that the *A. terreus* extract demonstrated a safe profile up to a dosage of 1 mL/kg. Furthermore, no signs of toxicity were seen during the 7 d of treatment.

Ethics approval

The experimental procedures followed the ethical principles and guidelines established by the Ethics Committee of the Faculty of Science, Al-Azhar University, Cairo, Egypt (AZHAR 2-03/2024). These guidelines are in line with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85–23, 1996), which ensures the proper use and welfare of experimental animals. The techniques and procedures used in this study adhere to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020).

CRediT authorship contribution statement

Amr M. Shehabeldine: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amer M. Abdelaziz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mostafa A. Abdel-Maksoud:** Writing – review & editing, Funding acquisition, Formal analysis. **Mohamed A. El-Tayeb:** Writing – review & editing, Funding acquisition, Formal analysis. **Bushra H. Kiani:** Writing – review & editing, Investigation. **Ahmed S. Hussein:** Investigation, Resources, Data Curation, Writing—original draft preparation, Writing—Review and Editing and Supervision.

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Conflict of interest

The authors declare that they have no conflict of interests.

Supplementary material

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Data availability

Data will be made available on request.

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