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In vitro assay and GC-MS profiling of Proteus sp.EKWG2I3 isolate obtained from Echinops kebericho Mesfin: A traditional medicinal plant

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Abstract

Endophytic bacteria are a promising source of bioactive compounds valuable to medicine, agriculture, and industrial applications. This study investigates bioactive compounds of EKWG2I3 to evaluate their *in vitro* antibacterial and antioxidant activity against test strains. A healthy root of *Echinops kebericho* Mesfin medicinal plant was collected for experimental analysis. 16S rRNA gene sequencing was performed for accurate identification of a single potential isolate. *In vitro* antibacterial, antioxidant activities, and GC-MS profiling of crude extracts for EKWG2I3 isolate were performed. The potent isolate *(*EKWG2I3) obtained from *E. kebericho* Mesfin clustered with *P. mirabilis* ATCC 29906^T (ACLE01000013) and designated as *Proteus* sp. EKWG2I3. The results revealed that the crude extract of the *Proteus* sp.EKWG2I3 isolate showed notable antibacterial activity of (15 ± 0 mm) against *E. coli* ATCC-25922^T. This isolate also exhibited potent free radical scavenging potential with an IC_{50} value of 11.98 µg/mL compared to ascorbic acid. Moreover, GC-MS analysis identified methyl phenylacetate (38.49%) and pentanoic acid, methyl ester (12.71%) as major compounds in the extract of *Proteus* sp.EKWG2I3. This study suggests that *Proteus* sp.EKWG2I3 isolate associated *Echinops kebericho* Mesfin could be a potential source of bioactive compounds to combat newly emerged diseases due to drug resistant pathogens.

1. Introduction

Endophyte is microbes that inhabit healthy plant tissues without causing any known effects or diseases against host plants (Egamberdieva et al. 2020; Rat et al. 2021). These endophytic bacterial species chiefly promote plant growth by producing phytohormones, siderophores, and mineral solubilization (Behera et al. 2017). They directly defend against phytopathogen by releasing secondary metabolites (Nxumalo et al. 2020), and also indirectly inducing host defense mechanisms (Yu et al. 2016). These endophytes can enhance induced systemic tolerance in host plants against different abiotic stresses, such as drought (Lim and Kim 2013; Shiva et al. 2016) and salt concentration (Dubey et al. 2020). Furthermore, they enhance disease resistance against various plant pathogens, such as *Pseudomonas syringae* pv. tomato DC3000 (Qu et al. 2021), *Ralstonia solanacearum* (Snoeck et al. 2022).

Biofilm formation in endophytic bacteria is employed during root colonization (Liu et al. 2011). Under stress conditions, biofilm formation emerges as a crucial strategy for plant growth promoting bacteria to thrive in the complex and dynamic plant environment. It provides plant-associated bacteria with resilience and adaptability through persistence, and colonization, and safeguards the bacteria, which in turn facilitates nutrient solubility in the intricate environment (Iungin et al. 2024). Moreover, endophytic bacteria also exhibited ACC deaminase secretion, which is considered plant plant-promoting catalytic enzyme. It catalyzes the degradation of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, into α-ketobutyrate and ammonia which in turn reduces the inhibitory effects of elevated levels of ethylene (Aswathy et al. 2014).

Endophytic bacteria associated with medicinal plants are a potential source of untapped bioactive compounds (Singh et al. 2017; Nxumalo et al. 2020).They produce a plethora of secondary metabolites with unique structures and profound biological activities and significant pharmaceutical potentials such as antioxidant (Photolo et al. 2020; Lata and Gond 2025), anticancer (Maela et al. 2022; Damavandi et al. 2023), immunomodulatory (Pandey et al. 2018), and antiviral. The secondary metabolites produced by these

endophytes are analogous to those of the host plant or non-analogous and are recognized as promising sources of biologically active compounds for drug development (Falade et al. 2021). These metabolites comprise essential enzymes, alkaloids, flavonoids, phenolic acids, quinines, steroids, and terpenoids (Zekry et al. 2019; Maela et al. 2022). Although these metabolites were primarily beneficial to the host plant, they have found a wide-ranging application industrially. They are harvested and used as agrochemicals, antibiotics, immunosuppressants, and antioxidants (Nxumalo et al. 2020). Diversity of endophytic bacterial genera such as *Bacillus*, *Enterobacter*, *Pseudomonas*, *Rhodococcus*, *Rhizobium*, *Serratia* sp., and others has been reported from ethno-medicinal plants (Rustamova et al. 2020a; Adeleke et al. 2022). Among them, most dominant genera belongs to *Bacillus, Burkholderia*, and *Pseudomonas* (Rana et al. 2020).

A rapid growth of drug-resistant microbes, discovery of new cases of life-threatening infections, and constant recurrence of diseases have pushed for advances in the field of drug discovery (Alvin et al. 2014). Rarely explored endophytes are currently recognized as a potential source for the discovery of novel natural products, as they participate in the biochemical pathways of their hosts and produce analogous or novel bioactive compounds (Chatterjee et al. 2019). *Echinops kebericho* Mesfin (Asteraceae Family) is an Ethiopia's well-known endemic medicinal plant (Ameya et al. 2016; Deyno et al. 2021; Tafesse et al. 2023). This medicinal plant has traditionally been used to treat infectious and non-infectious diseases (fever, headache, stomachache, and cough) (Toma et al. 2015; Ameya et al. 2016). Previous studies on phytochemical analysis of root extracts of this plant showed the presence of various antimicrobial chemical compounds (Ameya et al. 2016; Deyno et al. 2021; Agidew 2022). However, the study of endophytic bacterium *Proteus* sp.EKWG2I3 isolate, associated with *Echinops kebericho* Mesfin, an endemic medicinal plant, as potential sources of bioactive compounds is unexplored areas in Ethiopia. Therefore, this study aimed to investigate the bioactive compounds of *Proteus* sp.EKWG2I3 isolate with their *in vitro* biological activity against selected test pathogens.

2. Material and Methods

2.1. Plant material collections

Healthy roots of *E. kebericho* Mesfin (Fig. 1) was collected from Wondo Genet Agricultural Research Center (WGARC). WGARC is located at 7^{o} 192 N latitude and 38^{o} 382 E longitude with altitude of 1780 m a.s.l. It receives mean annual rainfall of 1128 mm with minimum and maximum temperature of 11.47 and 26.51^{o}C, respectively. The soil textural class of the experimental area was sandy loam with the pH of 6.4 (Mola and Kebele 2024). A total of eight *E. kebaricho* mesfin were collected separately and transported to Adama Science and Technology University using plastic bags, and stored at 4^{o}C for further analysis (Chen et al. 2022).

2.2. Sample processing methods

All the collected samples were washed thoroughly using tap water and air-dried (Toghueo et al. 2017). Then, each sample was surface sterilized by dipping them in 75% (v/v) ethanol for 5 min, followed by dipping in sodium hypochlorite (NaOCl) (2%, v/v) for 5 min and subsequently rinsed in sterile distilled water (Kumar et al. 2016; Rustamova et al. 2020a). The excess water was dried under a laminar airflow chamber (Saini et al.

2016). To check for surface infection, 0.1 ml of rinsing water from the last washing was plated onto nutrient agar (NA). The plates were incubated at 30°C for 5 days and observed for bacterial growth (Nxumalo et al. 2020).

2.3. Screening of endophytic bacteria isolates

Nutrient agar supplemented with Ketoconazole at 30 µg/mL was used to identify the EBIs. 1 g of sterilized samples was ground using a sterile mortar and pestles (Mortar and Pestel, 4 Oz, USA) and suspended into 9 ml (0.9% w/v) sterile saline solution (NaCl) under aseptic conditions. The sample extracts were serially diluted (10^{-4}) in a sterile saline solution (Egamberdieva et al. 2020). 100 µl of each diluted sample was pipetted on to Nutrient agar plates and spread evenly using a sterilized glass spreader. All inoculated plates were incubated at 30°C for 48 h. The grown colonies were selected based on divergence in morphology, size, colour and other features. These colonies were further purified on NA and stored at 4°C (Rustamova et al. 2020b).

2.4. Detection of ACC deaminase producing endophytic bacterial isolates

The ACC deaminase was performed following the methods of (DWORKIN and FOSTER 1958). Briefly, newly obtained endophytic bacterial isolates were inoculated on DF salt minimal medium (g/l) (KH_2PO_4, 4.0 g; Na_2HPO_4, 6.0 g; $MgSO_4.7H_2O$, 0.2 g; glucose, 2.0 g; gluconic acid, 2.0 g; citric acid, 2.0 g and agar, 12 g with trace elements; $FeSO_4.7H_2O$, 1 mg; H_3BO_3, 10 µg; $MnSO_4.H_2O$, 10 µg; $ZnSO_4.7H_2O$, 70 µg; $CuSO_4.5H_2O$, 50 mg; 10 µg MoO_3 amended with 2 g/l $(NH_4)_2SO_4$. The presence of bacterial growth in the media after 48 h was considered as a positive result for ACC deaminase test (Aswathy et al. 2014).

2.5. Detection of biofilm formation in endophytic bacteria isolates

A total of 20 EBIs were subjected to biofilm detection methods described by (Luu et al. 2021) with slight modifications. A loopful of overnight grown EBIs was inoculated in 10 ml of LB broth in test tubes. All these test tubes were incubated at 30°C for 96 h. After incubation, tubes were decanted and washed with phosphate-buffered saline (pH 7) and dried. All test tubes were then stained with crystal violet (0.1%, w/v). The excess stain was washed using deionized water. Tubes were dried in an inverted position. Finally, biofilm formation was considered positive when a visible film lined the wall of test tubes. Biofilm formation was scored as weak/none, moderate, and high/strong. The experiment was performed in triplicate.

2.6. A 16S rRNA gene sequencing for *Proteus* sp.EKWG2I3

Accurate identification of the endophytic bacterial isolates was carried out using 16S rRNA gene sequencing (Liu et al. 2017). *Proteus* sp.EKWG2I3 isolate was chosen for sequencing based on plant promoting traits such as biofilm formation and ACC deaminase enzymes production. Genomic DNA was extracted, and its quality was checked using (1.8%) agarose gel electrophoresis. The 16S rRNA gene was amplified using universal primers: 27F (GAGTTTGATCATGGCTCAG) and 1492R (TACGGTTACCTTGTTACGACTT). Sequencing of the amplified 16S rRNA gene was performed using the BDT v3.1 Cycle Sequencing Kit on an ABI 3500Dx Genetic Analyzer. Moreover, phylogenetic tree was constructed by Mega version 11 software and *Streptococcus agalactiae* ATCC 13813^T was served as an out group species.

2.7. Extraction of bioactive compound from *Proteus* sp.EKWG2I3 isolate

Bioactive metabolite extraction from *Proteus* sp.EKWG2I3 isolate (originated from roots of *E. kebericho* Mesfin) was performed according to previously described method by (Nxumalo et al. 2020). The secondary metabolites were extracted by inoculating 100 µl of overnight inoculum of isolate (24 h at 30°C) into 500 ml of LB broth. The culture cell was incubated on a rotary shaker at 30°C, 130 rpm for 5 days. After fermentation, the broth culture was centrifuged at 5000 rpm for 30 min. The supernatant was collected and the pellet was discarded. The supernatant was mixed with an equal volume of ethyl acetate (500 ml) and then left overnight at 4°C. The solvent phase containing secondary metabolites was separated using separatory funnel and concentrated using rotavapor at 45°C with 90 rpm. The complete dried crude extract was weighed and stored at 4°C for further analysis.

2.8. GC-MS analysis of ethyl acetate crude extract

GC-MS analysis of ethyl acetate crude extracts of endophytic bacterial isolate, *Proteus* sp.EKWG2I3, was performed using GC-MS, (7890B/5977A, Agilent Technologies, USA). Helium was used as carrier gas and flow rate adjusted at 1 mL/min. The GC had an HP-5MS column (non-polar column, Agilent Technologies), 30 m × 250 µm internal diameter, and 0.25 µm film thickness. The injector temperature was 260°C, and the injection mode was split mode with a split ratio of 20:1. The initial oven temperature was programmed from 40 ˚C and held for 2 min. It was raised to 150 ^{o}C at 15˚C/min at this temperature held for 1 min and then ramped by 30 ˚C/min to reach 300 ˚C at this temperature held for 5 min. Mass spectra were recorded in EI mode at 70 eV with scanning range in between 45–500 m/z. The secondary metabolites were identified by comparing their spectra with those in the NIST11 GC-MS library (Panigrahi and Rath 2021). Retention indices (RIs) were calculated relative to a C7–C40 n-alkane standard mixture run under the same GC temperature program.

2.9. Antibacterial activity of *Proteus* sp.EKWG2I3 isolate

Antibacterial activity of crude extracts of *Proteus* sp.EKWG2I3 isolate was performed using Mueller Hinton Agar medium (MHA) following previous protocols (Nxumalo et al. 2020; Nyalo et al. 2023). It was evaluated using gram-positive bacteria (*S. aureus* ATCC-25923^{T} and *S. pyogenes* ATCC-19615 ^{T}) and gram-negative bacteria (*E. coli* ATCC-25922 ^{T} and *P. aeruginosa* ATCC-27853 ^{T}) brought from Ethiopian Public Health Institute (EPHI). Moreover, Ciprofloxacin (30 µg) was used as positive control. Briefly, 10 µL of each tested strain were transferred to a Muller Hinton broth aseptically and incubated at 37°C for 24 h. The turbidity of the suspension was adjusted to 0.5 McFarland standards. The serial dilution of the extract was performed as 1000, 500, 250, and 125 µg/ mL using (4%) DMSO as the diluting agent. Each pathogen strains were swabbed on sterile MHA plates. Impregnated discs (6 mm) of each serially diluted extract, positive and negative controls (4% DMSO) were placed on inoculated MHA and left for 15 min. After complete diffusion, all plates were incubated at 37°C for 24 h. Finally, zones of inhibitions (mm) were recorded and reported as mean ± SD.

2.10. Antioxidant activity of *Proteus* sp.EKWG2I3 isolate

The free radical scavenging activities of Ethyl acetate crude extracts of EBIs were performed using *in vitro* DPPH assay according to (Nxumalo et al. 2020). Serial dilution of EBIs crude extract was prepared at 1000,

500, 250, 125, and 62.5 µg/mL using methanol (CH_3OH). The DPPH (0.04% w/v in methanol) solution was mixed (1:1 v/v) with the extract at different concentrations and kept at room temperature in the dark for 30 min. A sample-free DPPH solution was used as a negative control whereas ascorbic acid (AA) served as a positive control. The absorbance was measured using a UV-Vis spectrophotometer at 517 nm by using sample-free DPPH solution as a negative control. The percentage of DPPH radical scavenging activity (RSA) was calculated using the following formula in Eq. 1:

%DPPH scavenging activity =
$$\raisebox{1ex}{\$\text{A control} - \text{A sample}\$}/\raisebox{-1ex}{\$\text{A control}\$} \; x \; 100 \quad (1)$$

Where, $A_{control}$ is the absorbance of DPPH, and A_{sample} is the absorbance of the sample with DPPH solution. The inhibitory concentration (IC_{50}), the concentration needed to scavenge 50% of DPPH radical was calculated from the relationship curves (Abera et al. 2024).

2.11. Nucleotide sequence accession number

The 16S rRNA gene sequencing of *Proteus* sp.EKWG2I3 isolate was deposited in the GenBank with PV635194 accession number.

2.12. Statistical analysis

The experimental data were input into Microsoft Excel to calculate the mean inhibition zone and scavenging activity of the *Proteus* sp.EKWG2I3 crude extract. Additionally, the data were further analyzed and visualized using the latest version of Origin 2021 software. All reported data represent the mean of experiments conducted in triplicate.

3. Results and discussion

Endophytic bacterial isolates (EBIs) with various features had been screened from *E. kebericho* Mesfin. Some of them can produce ACC deaminase and other can form biofilms. Some of these EBIs were able to utilize a carbon sources (i.e. fructose, glucose, maltose, and sucrose), which supposed to enhance their growth. Among screened EBIs obtained from *E. kebericho* Mesfin, (75%) were capable to produce a biofilm. Some of these EBIs still could not produce biofilm (25%). The highest biofilm formations (25%) of EBIs were obtained from *E. kebericho* Mesfin (Table 1). Similarly, (Luu et al. 2021) confirmed the formation of various amounts of biofilm among endophytic bacteria. Previous studies also revealed that strong (64 isolates), moderate (8 isolates), and non-biofilm producers (20 isolates) were reported EBIs from *Clerodendrum colebrookianum* (Abera et al. 2024). (Hazarika et al. 2021) was also revealed endophytic isolates such as *Stenotrophomonas* sp. and *Pseudomonas* sp. were strong biofilm former which aids in root colonization and plant growth promotion in *Camellia sinensis*.

Table 1
Biofilm and ACC deaminase enzyme synthesis against the recent EBIs, these were collected from the medicinal plant, *E. kebericho* Mesfin.

S.N	EBIs	Biofilm	ACC deaminase	Maltose	Fructose	Sucrose	Glucose
1	*P. mirabilis* EKF6I1	+++	-	-	-	-	-
2	*Pseudomonas* sp. EKF5I1	+++	+	-	+	+	+
3	*Bacillus* sp.EKF3I1	-	-	-	-	-	+
4	*B. cereus* EKF1I7	+	-	-	-	-	+
5	*B. cereus* EKF1I2	++	-	-	-	-	-
6	*B. cereus* EKF3I5	-	-	-	-	-	+
7	*Proteus* sp.EKWG2I3	+++	+	+	+	+	+
8	EKF3I2	-	-	-	-	-	+
9	EKWG1I1	-	+	-	+	+	+
10	*Salmonella* sp.EKWG1I3	+++	+	-	+	-	+
11	*Pseudomonas* sp.EKWG2I1	++	+	-	+	+	+
12	*Bacillus* sp.EKF1I3	++	-	-	-	-	+
13	*B. cereus* EKF3I6	+	-	-	-	-	+
14	*B. cereus* EKF6I2	++	-	-	-	-	+
15	*Bacillus* sp.EKF1I5	++	-	-	-	-	+
16	EKF5I2	+	+	-	-	-	+

Key note: +: positive result, -: negative result, +++: high, ++: moderate. In this table, **P. mirabilis EKF6I1**, **Pseudomonas sp. EKF5I1**, **Proteus sp.EKWG2I3, and Salmonella sp.EKWG1I3 displayed the highest biofilm formers. Six EBIs are found to produce ACC deaminase. Fourteen EBIs were biofilm producers. Proteus sp.EKWG2I3 is the only isolate to utilize Maltose as a sole carbon sources.**

The recent EBIs (6, 37%), which are associated with *E. kebericho* Mesfin tend to secrete 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzymes. Among these isolates, *Proteus* sp.EKWG2I3, *Salmonella* sp. EKWG1I3 and *Pseudomonas* sp.EKWG2I1 were displayed significant prospects for ACC deaminase in addition to other biochemical features (Table 1). In line with this study, seven ACC deaminase enzymes producing endophytic bacteria, which are associated with *Commelina communis* plants, had been screened and characterized in China (Shin et al. 2012). Another study showed that (Hazarika et al. 2021) 87.73% of endophytic bacteria species were found to produce ACC deaminase enzymes, which is much higher than the

present result. Moreover, (Ali et al. 2014) also showed that drought-tolerant bacterial strains were potential ACC deaminase enzyme producers.

In this study, 16S rRNA gene sequencing was performed for potential isolate, *Proteus* sp.EKWG2I3, based on its ability to produce biofilm and ACC deaminase enzyme (Table 2). Phylogenetic analysis based on 16S rRNA gene sequences revealed that *Proteus* sp.EKWG2I3 (PV635194, 1110 bp) clustered within the *Proteus* clade and showed the closest relationship with *Proteus mirabilis* ATCC 29906^T (ACLE01000013) (99.99% site coverage), supported by a bootstrap value of 85%, a good bootstrap support. This indicates that it is moderate to strong confidence in this association, likely the same species (> 98.7 %) (Sakamoto and Benno 2006). *Proteus* sp.EKWG2I3 (PV635194, 1110 bp) is also clustered closely with others *Proteus* and *Xenorhabdus* strains (Fig. 2) with various frequencies. For instance, *Proteus* sp.EKWG2I3 has 99.91% closest sequence similarity to the *Proteus mirabilis* ATCC 29906^T (ACLE01000013) with 94.3% sequence completeness (Fig. 2, Table 2) and various GC%.

Table 2
Sequence similarities between *Proteus* sp.EKWG2I3 & related strains extracted from NCBI database

S.N	Sequence	Strain	Accession	Seq. similarity	Completness	GC%
1.	*Proteus* sp.	EKWG2I3	PV635194	---	---	52.75
2.	*P. mirabilis*	ATCC 29906^T	ACLE01000013	99.91	94.3%	53.11
3.	*P. columbae*	08MAS2615^T	MF143629	99.54	94.3%	53.55
4.	*P. vulgaris*	ATCC 29905^T	DQ885257	99.37	100.0%	53.07
5.	*P. cibi*	FJ2001126-3^T	MG913248	99.37	97.2%	53.16
6.	*P. penneri*	NCTC 12737^T	DQ885258	99.37	93.7%	53.28
7.	*P. alimentorum*	08MAS0041^T	KY930948	99.35	94.3%	53.12
8.	*P. faecis*	TJ1636^T	MG269469	99.17	94.3%	53.22
9.	*P. terrae* sub sp. cibarius	JS9^T	FJ796245	99.10	99.9%	53.42
10.	*P. terrae* sub sp.terrae	N5/687^T	LN680103	99.01	98.6%	53.29
11.	*P. hauseri*	ATCC 700826^T	LXEV01000040	98.65	100.0%	53.24
12.	*P. myxofaciens*	ATCC 19692^T	LXEN01000172	98.29	100.0%	53.79
13.	*X. hominickii*	KE01^T	DQ211719	97.48	99.9%	55.00
14.	*X. khoisanae*	SF87^T	HQ142625	96.76	97.2%	55.55
15.	*X. thuongxuanensis*	30TX1^T	MKGR01000070	96.40	100.0%	55.84
16.	*X. innexi*	DSM 16336^T	AJ810292	96.22	100.0%	55.29
17.	*X. stockiae*	TH01^T	DQ202309	96.13	100.0%	55.12
18.	*X.aichiensis*	XENO-7^T	OQ439939	96.09	93.1%	55.06
19.	*X. vietnamensis*	DSM 22392^T	MUBJ01000075	96.04	100.0%	56.38
20.	*X. yunnanensis*	XENO-10^T	JAQRFI010000151	96.04	100.0%	56.25
21.	*X. ehlersii*	DSM 16337^T	AJ810294	95.95	100.0%	55.29

S.N	Sequence	Strain	Accession	Seq. similarity	Completness	GC%
22.	*X. poinarii*	DSM 4768^T	X82253	95.93	100.0%	55.37

Key note: GC%: Guanine and Cytosine percentage. Table 2 **indicates comparative 16S rRNA gene sequence analysis of Proteus sp. EKWG2I3 and related reference strains of the genera Proteus and Xenorhabdus with their respective GenBank accession numbers, percentage sequence similarity, sequence completeness, and GC content (%).**

As shown in Table 2, *Proteus* sp.EKWG2I3 (PV635194) shared the lowest sequence similarity (95.93%) with *Xenorhabdus poinarii* DSM 4768^T (X82253) obtained from the database. The GC content of 16S rRNA gene sequence was found to be 52.75% for the recent isolates and other strains which shared sequence similarity with *Proteus* sp.EKWG2I3 has more than 52.75 GC% (Table 2). Consistently, (Egamberdieva et al. 2020) identified 11 genera of endophytic bacteria from horseradish plants using 16S rRNA gene sequencing. (Duhan et al. 2020) was also identified endophytic bacterial genera, associated with *Tinospora cordifolia* medicinal plant based on 16S rRNA gene analysis as *Bacillus, Aneurinibacillus* and *Pseudomonas*. Furthermore, *B. safensis* and *B. subtilis* were also reported from *Salvodora oleoides*, *Commiphora wightii*, and *Grewia tenax* medicinal plants relying on 16S rRNA gene sequencing (Priyanka et al. 2018).

The newly identified *Proteus* sp.EKWG2I3 endophytic bacterial isolate was obtained from *E. kebericho* Mesfin, medicinal plant. The ethyl acetate crude extract of *Proteus* sp.EKWG2I3 isolate detected with various secondary metabolites using GC-MS tools (Additional File: Table S_1, Fig. 3). Among identified compounds, methyl phenylacetate (38.49%) was the most abundant followed by Pentanoic acid, methyl ester (12.71%). However, Benzene acetic acid was found to be less abundant (0.23%) compound in this study. Inline to this study, (Panigrahi and Rath 2021) was identified various secondary metabolites from *Enterobacter cloacae* (MG001451), EBIs obtained from *Ocimum sanctum*. Moreover, previous (Nxumalo et al. 2020) study also demonstrated that dioctyl phthalate (50.51%) and (1, 2, 4) oxadiazole, 5-benzyl-3 (10.44%) a major compound from crude extract of *P. aeruginosa* CP43328.1. Methyl phenylacetate, a bioactive compound from *Bacillus mycoides* BM02, were identified for their ability to suppress plant diseases (Wu et al. 2020). Previous study also revealed that endophytic fungal extract comprises various bioactive compounds with a wide spectrum of antimicrobial activity (Maela et al. 2022). Furthermore, (Seenivasan et al. 2022) also reported 2,4-Di-tert-butyl-phenol as a principal secondary metabolite of endophytic *Streptomyces* sp. KCA1 1 of *Phyllanthus niruri*, which possesses antibacterial and anticancer properties.

Ethyl acetate crude extract of *Proteus* sp.EKWG2I3 isolate was shown various zone of inhibition (ZI) against test pathogens using disc diffusion method. At highest concentration (1000 µg/mL), crude extract of *Proteus* sp.EKWG2I3 isolate was shown the highest ZI, (15 ± 0 mm) against *E. coli* ATCC-25922 ^{T}, whereas least inhibitory effect was detected against *P. aeruginosa* ATCC-27853 ^{T} with ZI (10 ± 0.01 mm) compared to ciprofloxacin with (21 ± 0.05) (Table 3, Fig. 4). Similarly, (Kiros et al. 2024) revealed that the highest inhibitory value (10.1 ± 0.1 mm) was recorded against *E. coli* ATCC-25922^T using n-hexane crude extract. In agreement with this study, (Mamarasulov et al. 2023) also reported highest antimicrobial activity of crude extracts of *B. subtilis* sp. (M10) (12 ± 1.4mm), *B. mojavensis* (M11) (8 ± 1.0 mm) and *B. amyloliquefaciens* (M13) (6.2 ± 0.3) against *E. coli* ATCC-25922 ^{T}. Further study noticed that (Kiros et al. 2024) n-hexane extract of *G. purpurascens*

used to give the highest inhibitory effect (10.1 ± 0.1 mm) for *E. coli*. The observed inhibition zone against test pathogens could be due to the presence of secondary metabolite such as Methyl phenylacetate, Pentanoic acid, methyl ester, 3-Pyridinecarbonitrile, 4-(methoxymethyl)-2-[(4-, Benzene ethanol ,1H-Indol-3-ylmethyl acetate, Phenol, 2,4-bis(1,1-dimethylethyl)- and other compounds in the present crude extract. These metabolites could target the pathogens' cellular structures like cell walls, cell membranes, protein synthesis, genetic materials, ribosomes, and other structures of the test pathogens.

Table 3
Antibacterial activity of crude extract of *Proteus* sp.EKWG2I3 against test strains

S.N.	**Conc. (µg /mL)**	***S. aureus* ATCC-25923^T**	***S. pyogenes* ATCC-19615 ^{T}**	***E. coli* ATCC-25922 ^{T}**	***P. aeruginosa* ATCC-27853 ^{T}**
		Zone of inhibition (mm)			
1	1000 µg/mL	12 ± 0	13 ± 0.05	15 ± 0	10 ± 0.01
2	500 µg/mL	10 ± 0	11 ± 0.01	12 ± 0.1	7 ± 0
3	250 µg/mL	8 ± 0	8.03 ± 0.05	9 ± 0.05	6.5 ± 0
4	125 µg/mL	7 ± 0	7 ± 0	8 ± 0.05	NA
5	Cipro. (30 µg/mL)	19 ± 0	21 ± 0	19 ± 0	21 ± 0.05

Key note: NA indicates No activity. Table 3 **displayed antibacterial activity of the test sample at different concentrations (µg/mL) against S. aureus ATCC 25923 T, S. pyogenes ATCC 19615 T, E. coli ATCC 25922 T, and P. aeruginosa ATCC 27853T, expressed as zone of inhibition (mm, mean ± SD), compared to ciprofloxacin (30 µg/mL) as a standard**

Table 4
Scavenging activity (%) of the ethyl acetate extracts of *Proteus* sp.EKWG2I3 isolates

S.N.	**(%, mean ± SD)**		
	Conc.(µg/mL)	***Proteus* sp.EKWG2I3**	**AA**
1	62.5	72.74 ± 0.01	76.54 ± 0.01
2	125	76.31 ± 0.02	80.94 ± 0.00
3	250	80.85 ± 0.02	86.17 ± 0.01
4	500	86.28 ± 0.01	90.86 ± 0.02
5	1000	88.13 ± 0.1	92.13 ± 0.01
6	IC_{50} (µg/mL)	11.98	3.49

Key note: AA indicates Ascorbic acid. Table 4 **is a concentration-dependent percentage (%) activity (mean ± SD) of the test sample against Proteus sp. EKWG2I3 in comparison with AA as a standard, along with IC_{50} values (µg/mL), and various corresponding concentrations.**

Our results have shown that the crude extract of *Proteus* sp.EKWG2I3 isolate displayed strong free radical scavenging activity (88.13 ± 0.1%) with an IC_{50} value of 11.98 µg/mL (Table 4, Fig. 5). Considerable amount of free radical scavenging activity (86.28 ± 0.01%) (Table 4) was also recorded for the crude extract of *Proteus* sp.EKWG2I3 at 500 500. The lowest free radical scavenging activity 72.74 ± 0.01% at 62.5 µg/mL, with 76.54 ± 0.01% corresponding to AA (Table 4). Similarly, (Photolo et al. 2020) reported that the *M. radiotolerans* MAMP 4754 extract, an endophytic bacterial isolate, was an effective free radical scavenger with an IC50 of 5.65 µg/mL. Additionally, a previous study (Manihot et al. 2024), showed that the BEDS3 isolate exhibited the highest antioxidant activity, with an IC50 value of 14.94 ppm.. (Demisie et al. 2024) It was also reported that *Bacillus* sp. Sl00103 crude extract demonstrated a notable antioxidant activity. Previous studies suggested that an extract with a low IC_{50} value is indicative of greater radical scavenging activity (Shahidi and Yeo 2018; Photolo et al. 2020). (Shahidi and Yeo 2018; Abdisa and Kenea 2021), and the GC- MS result confirmed the presence of phenolic derivatives in the crude extracts of *Proteus* sp.EKWG2I3 isolate. It was also revealed that a potential antioxidant activity of crude extract might be due to the presence of phenolic hydroxyl group in which the hydrogen atom can scavenge free radicals

4. Conclusion

Proteus sp.EKWG2I3 isolate, endophytic bacteria, was a potential source of bioactive compounds obtained from root of *E.kebericho* Mesfin. *In vitro* antibacterial assay showed that crude extracts of *Proteus* sp.EKWG2I3 isolate showed highest zone of inhibition (15 ± 0 mm) against *E. coli* ATCC-25922 ^{T} compared to ciprofloxacin. The BLAST analysis of the 16S rRNA gene sequencing of *Proteus* sp.EKWG2I3 isolate revealed 99.91% sequence similarity with *Proteus mirabilis* ATCC 29906^{T}. Based on GC-MS analysis, methyl phenylacetate (38.49%) was the most abundant bioactive metabolites in *Proteus* sp.EKWG2I3 isolate obtained from *E.kebericho* Mesfin, an Ethiopian traditional medicinal plant. Moreover, *Proteus* sp.EKWG2I3 extract exhibited a strong free radical scavenging effect with IC_{50} value of 11.98 µg/mL compared to Ascorbic acid. Based on this study, the bioactive compound-rich *Proteus* sp.EKWG2I3 isolate, an endophytic bacterium associated with *E. kebericho* Mesfin, an Ethiopian traditional medicinal plant, was explored for the first time. *In vitro* assay results could also suggests that bioactive compound obtained from *Proteus* sp.EKWG2I3 isolate aided to combat human diseases. Further study needs to focus on exploration of pure compounds from *Proteus* sp.EKWG2I3 bacterial endophyte and the elucidation their specific structures.

Declarations

Ethics approval and consent to participate

Echinops kebaricho Mesfin were collected according to national and institutional guidelines for plant research ethics. *Echinops kebaricho* Mesfin collection was done following legal procedure of institution, WGARC. During plant samples collection, no endangered and threatened species were employed.

Consent for publication

Not applicable.

Availability of data and materials

All data supporting the findings of this study are included within the article and supplementary materials

Competing interests

The authors declare that they have no competing interests. Adama Science and Technology University had no role in the development of the proposal, sample collection, laboratory work, data analysis, manuscript preparation, and the decision to publish the results. Therefore, all authors confirm that there are no competing interests.

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Authors contributions

Dinku Senbeta conducted experiments. Data analyses were conducted by Dinku Senbeta, and Seid Mohammed. The manuscript was prepared by Dinku Senbeta. Professor Aman Dekebo and Seid Mohammed supervised during laboratory experiments, and manuscript preparation. For this research, samples collection and laboratory analysis for biochemical tests and optimization were performed by Mr. Dinku Senbeta (MSc) under the supervision of Dr. Seid Mohammed (PhD) and Prof. Aman Dekebo (PhD). The major edition of manuscript was done by Dr. Seid Mohammed (PhD). However, the project was conceived, and designed Professor Aman Dekebo. The manuscript was finally checked by Prof. Aman Dekebo (PhD). All authors have approved the final version of the manuscript and agree to be held accountable for the content therein.

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Figures



Figure 1

(a) *E. kebericho* Mesfin, medicinal plant (b) Tuber of this plant

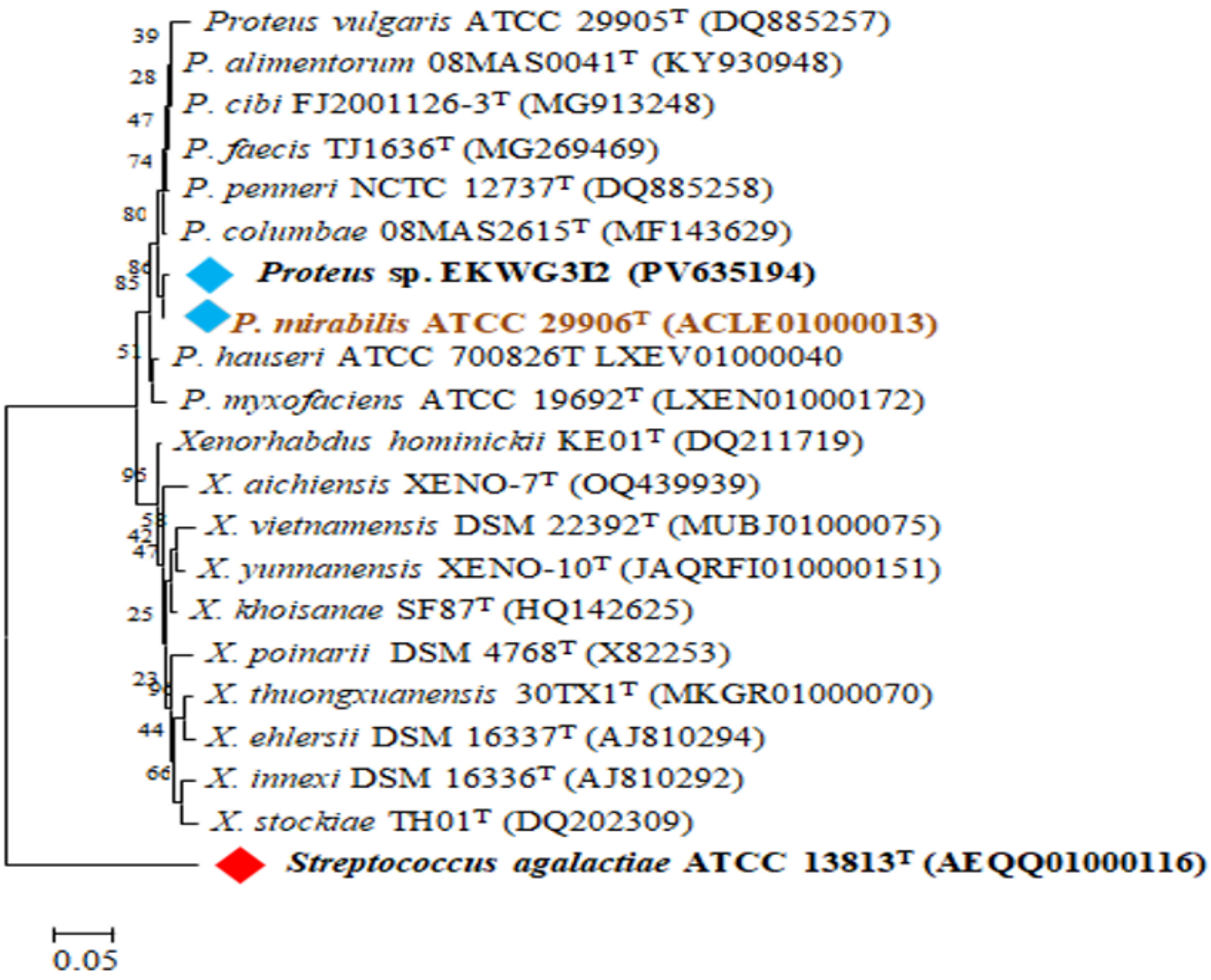


Figure 2

Evolutionary analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Jukes-Cantor model (Jukes and Cantor 1969)**. The tree with the highest log likelihood (-5058.74) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jukes-Cantor model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1480 positions in the final dataset. Evolutionary analyses were conducted in MEGA11** (Felsenstein 1985; Tamura et al. 2021)

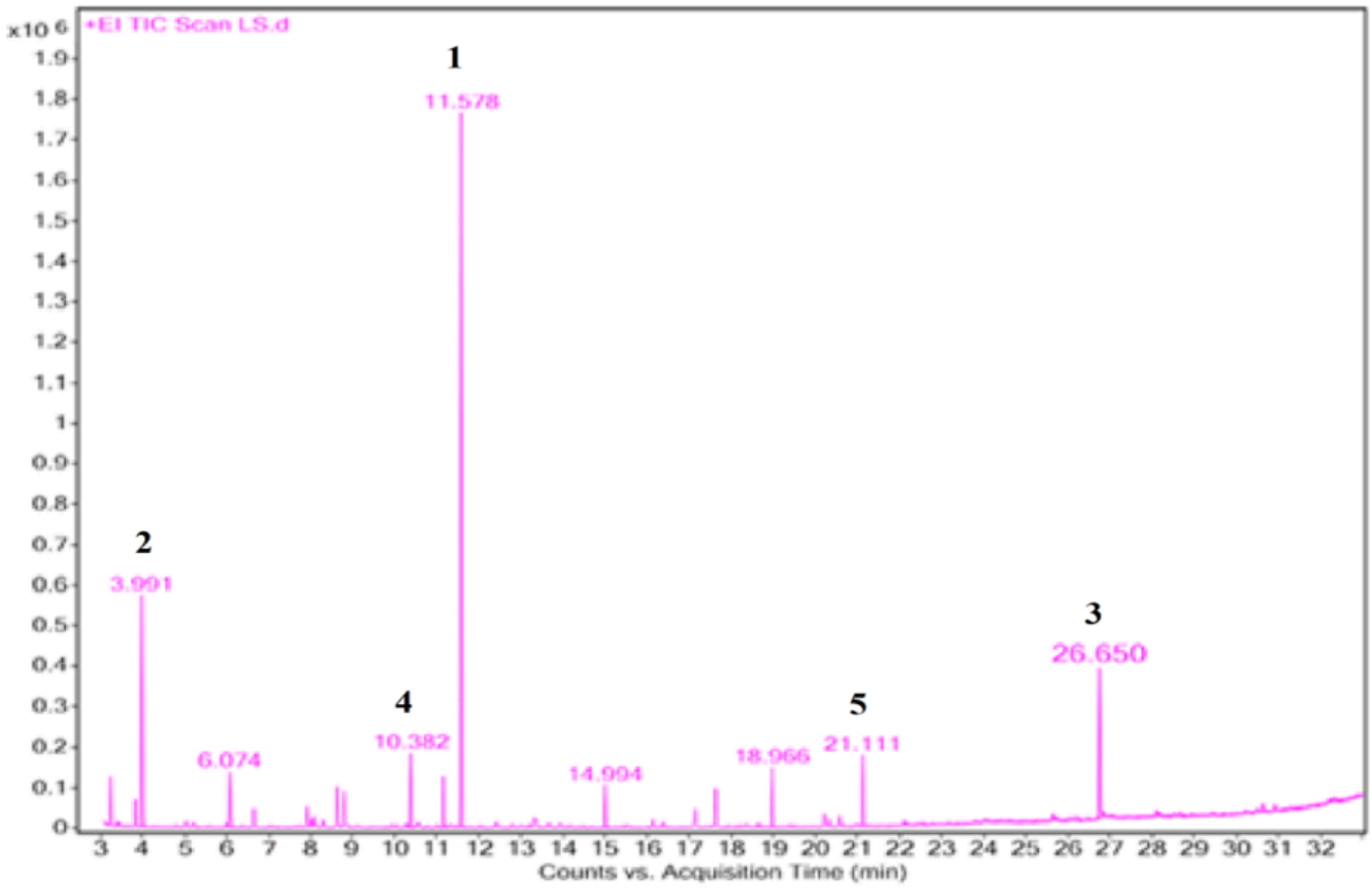


Figure 3

Gas spectrum of compounds from *Proteus* sp.EKWG2I3 isolate. The chromatogram exhibited 5 major peaks (1–5) and a few minor signals. Peak 1 is Methyl phenylacetate, Peak 2. is a Pentanoic acid, methyl ester, Peak 3 is 3-Pyridinecarbonitrile, 4-(methoxymethyl)-2-[(4-), Peak 4 is a Benzene ethanol and Peak 5 is a 1H-Indol-3-ylmethyl acetate.

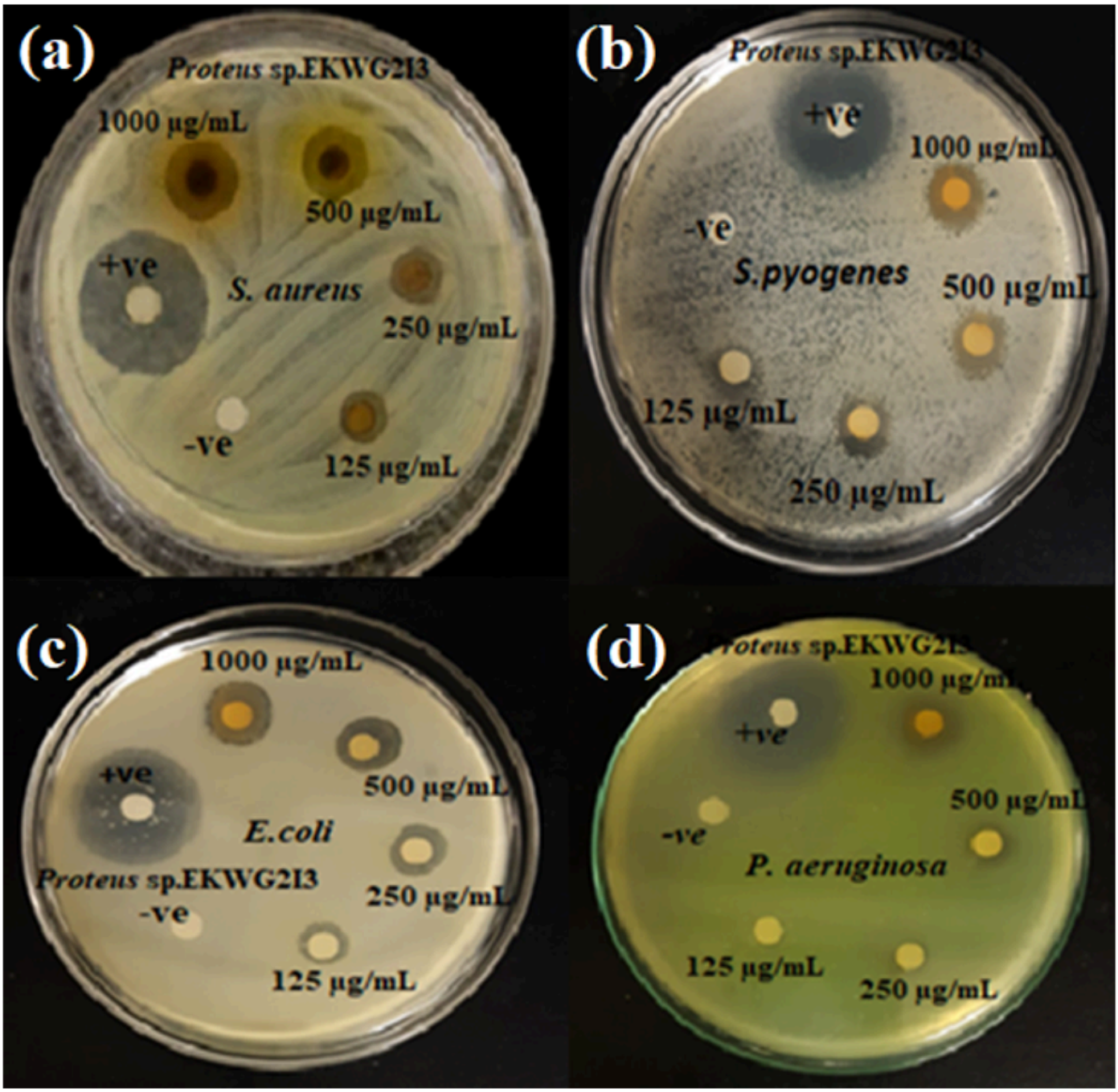


Figure 4

Antibacterial activity of various concentrations of crude extract for *Proteus* sp.EKWG2I3 against certain bacterial pathogenic strains. (a) Various concentrations of crude extract of *Proteus* sp.EKWG2I3 against *S. aureus* ATCC-25923^T. (b) Various concentrations of crude extract of *Proteus* sp.EKWG2I3 against *S. pyogenes* ATCC-19615^T. (c) Various concentrations of crude extract of *Proteus* sp.EKWG2I3 against *E. coli* ATCC-25922^T. (d) Various concentrations of crude extract of *Proteus* sp.EKWG2I3 against *P. aeruginosa* ATCC-27853^T.

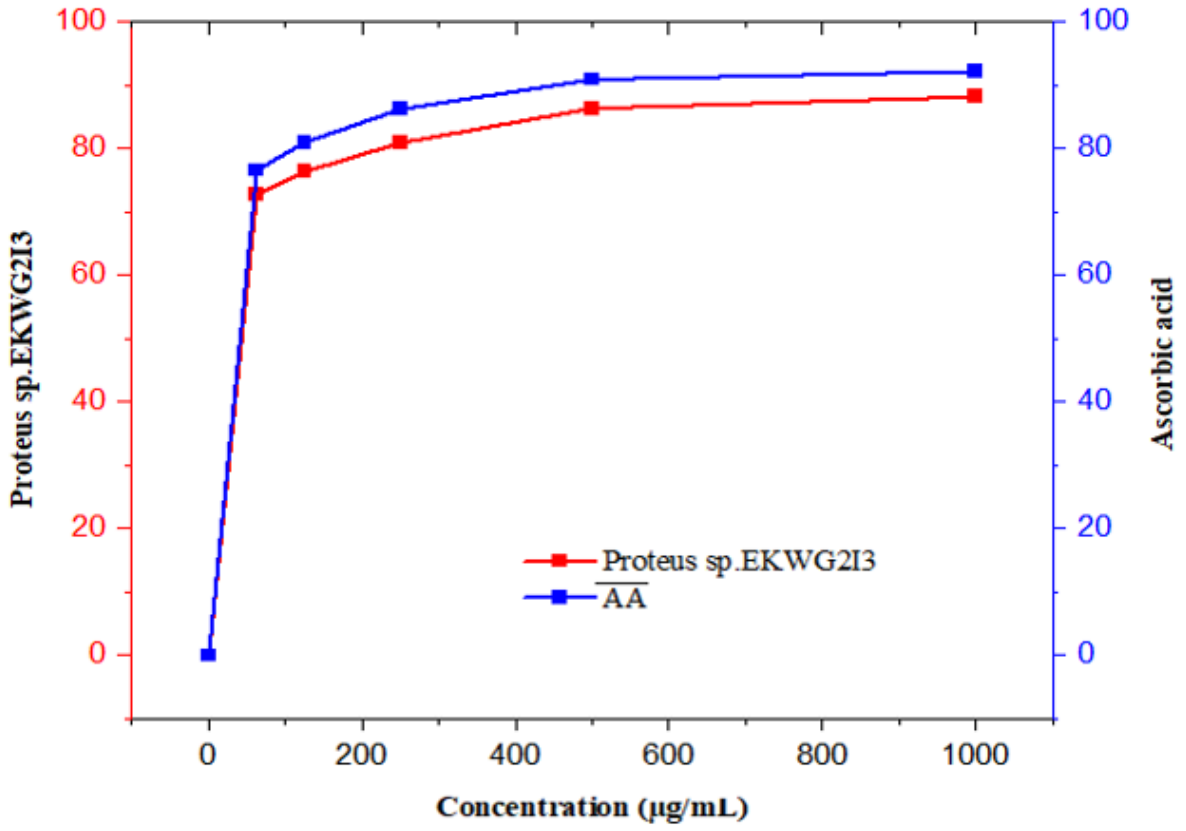


Figure 5

A DPPH activity of the crude extract of *Proteus* sp.EKWG2I3isolate. Dose–response relationship of *Proteus* sp. EKWG2I3 and ascorbic acid (AA) across various concentrations (0–1000 µg/mL). The graph shows an increase in activity for *Proteus* sp. EKWG2I3 (red line, left y-axis) and AA (blue line, right y-axis) with increasing concentration, reaching a plateau at higher concentrations (approximately ≥500 µg/mL), which supposed a saturation of response.

Supplementary Files

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