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Screening of an Actinobacterium *Streptomyces gilvifuscus* MKA19 for L-asparaginase production and to investigate its pharmacological properties

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ABSTRACT

Actinomycetes such as *Streptomyces* sp serve as a good source of L-asparaginase, a pharmacologically important enzyme for the treatment of many ailments. In this study, a mangrove associated actinobacterium, *Streptomyces gilvifuscus* MKA19 was screened for L-asparaginase production, and the produced enzyme was purified. The purified enzyme exhibited the specific activity of 4.70 IU/mg with 1.68-fold purification and the yield of 50.72%, with a molecular weight of 85 kDa. The antagonistic activity of the enzyme against human pathogens was evaluated by agar well diffusion method, anticancer activity of the enzyme against various cancer cell lines was evaluated by MTT assay and the *invitro* antioxidant activities (DPPH, hydroxyl radical scavenging and nitric oxide scavenging assays) of the enzyme were also evaluated. The results on the antagonistic activity of L-asparaginase against human pathogens exhibited the maximum zone of inhibition (25 mm) against *Streptococcus mutans* and *S. pneumoniae* at 40 µl concentration. The enzyme possessed significant inhibitory effect against MCF-7 cell line with the IC_{50} value of 105.681 µg/ml. The total phenolic content (GAE) of L-asparaginase was found to be 0.0352 mg/ml. The antioxidant properties of the enzyme by DPPH scavenging, Hydroxyl radical scavenging and Nitric oxide scavenging assays exhibited the IC_{50} value of 33.66, 271.67 and 221.31 µg/ml, respectively. From this study, it could be confirmed that the actinobacterium, *S. gilvifuscus* effectively produced the enzyme L-asparaginase with pharmaceutical potential.

Keywords: L-asparaginase, *Streptomyces gilvifuscus*, antimicrobial, anticancer, antioxidant

1. Introduction

Enzymes are proteins, which perform a variety of activities. They are biocatalysts produced by living organisms to catalyze certain biochemical reactions, which are typically part of the cell's metabolic activities. Enzymes can function intracellularly, extracellularly or even on the surface of a cell membrane (Maurer, 2001). Enzymes produced by microorganisms are used in a variety of enterprises around the world. More than 300 enzymes have been identified, with many finding their way into biotechnology and industrial uses. Among microbes, actinomycetes, particularly *Streptomyces* have been shown to produce enzymes such as amylase, lipase, protease, cellulase, chitinase, pectinase, insulinase, and glucose isomerase (Desai *et al.,* 2016). We require safer enzymes from less expensive sources; various studies have concentrated on the manufacture of enzymes by actinomycetes, particularly L- asparaginase (Bhargavi and Jayamadhuri, 2016). Marine microorganisms are regarded as untapped sources of metabolites and products with new characteristics. Marine actinomycetes are another excellent source of L-asparaginase (Narayana *et al.,* 1993).

Actinomycetes such as *Streptomyces griseus*, *S. karnatakensis*, *S. albidoflavus*, and *Nocardia spp*. can produce the enzymes (Narayana, 2008; Mostafa and Salama, 1979). Several *Streptomyces* species, including *S. karnatakensis*, *S. venuzuzlae*, *S. longsporuslavus*, *S. gulbargensis*, and marine *Streptomyces sp*. PDK2 have been investigated in regard to the production of L-asparaginase. Many methods have been established to screen bacteria for the synthesis of L-asparaginase (Mohamed *et al.,* 2015). The activity of L-asparaginase is accompanied by an increase in pH resulting from ammonia liberation; the presence of ammonia is used qualitatively and quantitatively to assess enzyme activity (Mashburn and Wriston, 1963; Abdelrazek *et al.,* 2019). Extracellular L-asparaginase is more easily produced and purified than intracellular enzymes (Desai *et al* 2018). Various purification procedures, including salt precipitation (Sahu *et al.,* 2007; Basha *et al.,* 2009) and column chromatography (Sahu *et al.,* 2007; Mohamed *et al.,* 2015; Ravi *et al.,* 2016), have been used during the purifying process.

Clinical uses of L-asparaginase indicated that it has strong antioxidant properties (Maysa *et al.,* 2010). Microbial L-asparaginase is frequently used as a treatment for human cancer (Giebel *et al.,* 2008). According to Wriston and Yellin (1973), L-asparaginase is commonly used as an anticancer agent; especially, Verma *et al.* (2007) and Marshall *et al.* (1999) reported L-asparaginase's anticancer impact in the treatment of acute lymphoblastic leukaemia. Few studies have consistently reported that L-asparaginase causes a wide range of adverse effects, including skin rashes, hepatic dysfunction, leukopenia, pancreatitis, hyperglycemia, seizures, and hemorrhage (Duval *et al.,* 2002). According to clinical investigations, bacterial endotoxins and enzyme glutaminase activity in enzyme preparations are the primary causes of L-asparaginase toxicity (Kotzia and Labrou, 2005). As such, purification of L-asparaginase is an essential step towards the physical and biological characterisation of the enzyme. Additionally, it is very desirable to employ novel L-asparaginase that differs serologically but yet has a comparable therapeutic impact (Tetsuya *et al.,* 1971). Therefore, it is still important to screen for novel organisms in order to find strains that can produce a large production of L-asparaginase while having less negative side effects (El-Naggar *et al.,* 2014). In due to this, the current investigation was conducted to assess the antibacterial, anticancer, and antioxidant qualities of the enzyme L-asparaginase, which was synthesized by a potent actinobacterium that was isolated from mangrove soil samples collected from Manakudy Estuary, Kanyakumari District, South India.

2. MATERIALS AND METHODS

2.1. Evaluation of *Streptomyces* strains producing L-asparaginase

The microbial culture collection of the Centre for Marine Science and Technology at Manonmaniam Sundaranar University in Rajakkamangalam contributed the producer strain of *Streptomyces gilvifuscus* MKA19 used in this investigation. The strain was first isolated from the sediment samples of Manakudy mangrove ecosystem, Kanyakumari District of South India. Initially, the modified Gulati *et al.* (1997) technique was used to screen the strain for the synthesis of L-asparaginase. The actinobacterial strain *S. gilvifuscus* MKA19 was streaked onto M9 agar plates, and the plates were then incubated at 28°C for seven days. The development of a pink zone surrounding the colonies served as confirmation of L-asparaginase synthesis during incubation.

2.2. Preparing the inoculum to produce L-asparaginase

Initially, *S. gilvifuscus* MKA19 was introduced to a freshly prepared ISP2 agar plate and cultured for five days at 28°C. Next, 20 ml of sterile ISP2 broth was aseptically inoculated with a loopful of this strain in an Erlenmeyer flask (100 ml), and the mixture was shaken continuously at 200 rpm for five days at 28°C. A 250 ml conical flask containing 100 ml of sterile M9 broth was filled with about 5% of the production inoculum. After that, the flask was incubated for five days at 28°C on a rotating shaker. Regular assays of the L-asparaginase production have been performed from the incubated broth. The medium was incubated, then fermented, and centrifuged for 20 minutes at 10,000 rpm to extract the crude enzyme, which was then used for further study (Dharmaraj, 2011).

2.3. L-asparaginase activity determination

Using the Nesslerization method, the activity of L-asparaginase from the culture-free supernatant was ascertained (Imada *et al.,* 1973). 0.5 ml of supernatant and 0.5 ml of L-asparagine (0.04 M) were added to a sterile test tube. The tube was then incubated in a water bath at 37°C for 30 minutes after adding 0.5 ml of 0.05 M Tris-HCl buffer (pH 7.2). To stop the reaction after incubation, 0.5 ml of 1.5 M TCA was added. 3.75 ml of sterile distilled water, 0.1 ml of the enzyme sample, and 0.2 ml of Nessler's reagent were added to a test tube in order to analyse the ammonia that was released from the reaction mixture. After a 10-minute incubation period at room temperature, the test tube's absorbance was measured at 450 nm using a UV-Vis spectrophotometer (Techcomp 8500). The amount of L-asparaginase that released one µmole of ammonia/ml/min was measured as one IU of activity.

2.4. Purification of L-asparaginase

The strain was centrifuged at 10,000 rpm and 4°C after its cultivation in the production medium for 72 hours. Following centrifugation, the crude enzyme source (supernatant) was extracted and subjected to a three-step purification procedure (Narayana *et al.,* 2008).

2.4.1. Ammonium sulphate precipitation

When adding ammonium sulphate to the 500 ml supernatant until it reached 80% saturation, the mixture was left to stand for the whole night. This solution was centrifuged for 15 minutes at 4°C at 10,000 rpm. Following centrifugation, the precipitate was collected and dissolved in a small volume of 50 mM Tris-HCl buffer (pH 7.2), with the supernatant being discarded.

2.4.2. Dialysis

Following precipitation, the collected precipitate was exposed to dialysis with a dialysis bag (12000 MW cut off) against three levels of 50 mM Tris-HCl buffer (pH 7.2) at 4°C in order to eliminate any excess salts.

2.4.3. Chromatography using anion exchange

In order to elute the bound enzyme, NaCl step gradients (0.2, 0.6, 0.8, and 1.0 M) in the same buffer were used to load the dialyzed protein on a DEAE-Sepharose fast flow chromatography (1.2 cm x 20 cm) (Sigma Aldrich, USA) that had been pre-equilibrated with three bed volumes of 50 mM Tris-HCl (pH 7.2). The flow rate for this process was 0.5

ml/min. Using a UV-Vis Spectrometer, the absorbance at 280 nm was used to track the elution. The total protein content and L-asparaginase activity of each of the collected fractions were next ascertained. Afterwards, Amicon Ultra centrifuge filters (10 kDa molecular weight cut off; Millipore Ireland Ltd, Ireland) were used to pool and concentrate the highly active fractions. The isolated L-asparaginase's molecular weight has been determined by SDS-PAGE (Laemmli, 1970) utilizing the Alphaimager mini gel documentation system (Cell Bioscience, USA).

2.5. Total Phenolic Content Determination

At room temperature, the assay mixture was incubated for 30 minutes. It contained 1.25 ml of Tris buffer (100 mM, pH 8.2), 0.5 ml of 1% aqueous casein solution, and 0.25 ml of culture supernatant. This mixture was then given 3 ml of 5% TCA to create a precipitate, and it was then refrigerated at 4°C for 15 minutes. After that, 2.5 ml of Na_2CO_3 (0.5M) was added to 0.5 ml of supernatant and allowed to keep at room temperature for 20 minutes. 0.5 ml of the Folin-phenol reagent was added to this mixture, and a UV-Vis spectrophotometer was used to measure the absorbance at 660 nm (Aisha *et al.,* 2022).

2.6. Determination of antibacterial activity of L-asparaginase

The antagonistic activity of L-asparaginase from *S. gilvifuscus* MKA19 was evaluated against human pathogens *viz*. *Streptococcus mutans* (NCIM 2063), *Escherichia coli* (MTCC96), *S. pneumoniae* (MTCC432), *Streptococcus aureus* (ATCC25928) and *Enterococcus faecalis* (ATCC29212) by agar well diffusion method. Wells of 5 mm diameter were made on Mueller Hinton Agar plates using sterilized cork borer and 0.1 ml each of the inoculum of test pathogens were spread individually on the agar surface. Different concentrations (from 10 to 40 µl) of the purified enzyme were added to the wells along with the positive control, chloramphenicol (25 mcg/ml). The plates were incubated at 37°C for 24 h and the zones of inhibition(mm) were measured (Valli *et al.,* 2012).

2.7. Evaluation of L-asparaginase's anticancer activity

The cytotoxic activity of L-asparaginase from *S. gilvifuscus* MKA19 was determined based on cell viability by adopting the MTT assay method using human cervical cancer (HeLa) (Fatima *et al.,* 2019), breast cancer (MCF-7) (Rani *et al.,* 2011) and human colon cancer (HT-29) (Dias *et al.,* 2016) cell lines obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The logarithmically growing selected cell lines were plated in flat-bottom 96-well microplate individually at a density of 1×10^3 cells and cultured for 24 h. Following that, pure L-asparaginase enzyme was added to different cell lines at various concentrations (100, 50, 25, 12.5, 6.25 µg/ml). After 24 h of incubation, 100 µg of 15 mg/ml MTT solution was added in to each well. Then, the cell lines were incubated in dark for 4 h in CO_2 incubator at 37°C. Following the incubation period, the supernatant was discarded, and 100 µg of MTT solubilization solution (DMSO) was added. The formazan crystals were then gently mixed into the wells. Through the use of a multi-well plate spectrophotometer, absorbance was measured at 540 nm. The results were calculated using the corresponding percentage of absorbance in relation to the control. Throughout the experiment, control (untreated cells) was also maintained. For every cell line, the experiment was carried out three times. Quercetin was used as a positive control to compare the results. Using a fluorescent microscope, pictures of each set of cancer cell lines that were treated were taken. The following formula was used to obtain the cell viability (%).

$$\text{The percentage cell viability (\%)} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

$$\text{The percentage of cell inhibition (\%)} = \frac{100 \text{ Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

2.8. The assessment of L-asparaginase's antioxidant activity

Using three different assays, the DPPH, hydroxyl radical, and the nitric oxide scavenging assay through the antioxidant potential of the L-asparaginase enzyme of *S. gilvifuscus* MKA19 was monitored.

2.8.1. DPPH scavenging assay

The DPPH assay was used to determine the purified enzyme's potential for radical scavenging (Chang *et al.,* 2007). Various enzyme concentrations (1.25, 2.50, 5.0, 10.0, and 20.0 µg/ml) were prepared in doses of 40 µl using DMSO. 2.96 ml of DPPH (0.1 mM) solution was then added, and the reaction mixture was allowed to stand at room temperature in the dark. Using a UV spectrophotometer, the mixture's absorbance was measured at 517 nm after 20 minutes. Simultaneously, a control with 3.0 ml of DPPH solution was used.

Inhibition rate (%) = [control-test/ control] × 100

2.8.2. Hydroxyl radical scavenging activity

With a few minor modifications, the Parejo *et al.* (2000) approach was used to carry out the hydroxyl radical scavenging activity. Fresh stock solutions of deoxyribose (10 mM), $FeCl_3$ (10mM), EDTA (1mM), ascorbic acid (1mM), and H_2O_2 (10mM) were made in distilled deionized water. The experiment was conducted by dissolving 1.0 ml of varying concentrations of purified enzyme (12.5, 25, 50, 100, and 200 µg/ml) in distilled water in each test tube. Next, 0.33 ml of phosphate buffer (50 mm, pH 7.4) and 0.1 ml of ascorbic acid were added. The experiment was completed by adding 0.1 ml of EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, and 0.36 ml of deoxyribose to each test tube. Subsequently, the mixture was incubated for one hour at 37ºC. 1.0 ml of the incubated mixture was mixed with 1.0 ml of TCA (10%) and 1.0 ml of TBA (0.5%) to create the pink chromogen and read at 532 nm. The enzyme's ability to scavenge hydroxyl radicals has been expressed as the percentage rate of inhibition (%) of deoxyribose degradation, which was determined using the following formula:

Inhibition rate (%) = [ABS (Control) – ABS (Standard) / ABS (Control)] × 100

ABS (Control) – absorbance of the control reaction

ABS (Standard) – absorbance of the standard reaction

2.8.3. Nitric Oxide scavenging assay

The test for nitric oxide scavenging was determined using a slightly modified version of Green *et al.* (1982) method. Each test tube with 0.5 ml of various doses of pure enzyme (12.5, 25, 50, 100, and 200 µg/ml) were taken along with 0.1 ml of sulphosalicylic was acid. The tubes were well vortexed for 30 minutes. For fifteen minutes, the samples were centrifuged at 5,000 rpm. 200 µl of the supernatant solution was obtained after centrifugation, and it was well mixed with 30 µl of 10% NaOH and 300 µl of Tris-HCl buffer. After adding 530 µl of Griess reagent to this mixture, it was left in the dark for ten to fifteen minutes. Then the reaction mixture was read at 546nm against Griess reagent as blank and sodium nitrate as the standard.

2.9. Statistical analysis

The data used in the current study were reported as Mean ± SD. One-way ANOVA test was used for analyzing the data, and STATISTICA 6.0 (Statosoft, Bedford, UK) was used to carry out a *post hoc* multiple comparison with SNK test at a 5% level of significance.

3. RESULT

3.1. Screening of L-asparaginase producing strain

The production of L-asparaginase enzyme was assessed in the strain *S. gilvifuscus* MKA19 by directly observing the pink zone surrounding the colonies in M9 medium plates, as illustrated in Fig. 1.

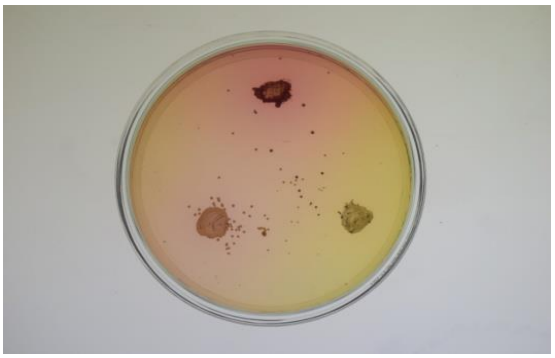


Fig. 1. L-asparaginase producing strain in M9 medium

3.2. Determination of L-asparaginase activity

L-asparaginase was produced further using the strain *S. gilvifuscus* MKA19. The production medium was centrifuged at 10,000 rpm for 15 minutes after 48 hours, and the supernatant was used as a source of crude enzyme. It was found that the enzyme had a specific activity of 2.78 IU/mg protein.

3.3. Purification of L-asparaginase

Several purification steps, including 80% ammonium sulphate precipitation, dialysis, and anion exchange chromatography, were applied to the collected supernatant (100 ml). Out of all the fractions that were collected, the 16th and 17th fractions exhibited the highest level of enzyme activity. Fig. 2 illustrates the highest L-asparaginase activity, however, in the elution profile from the DEAE-Sepharose rapid flow chromatography, which yielded a single peak (17th fraction).

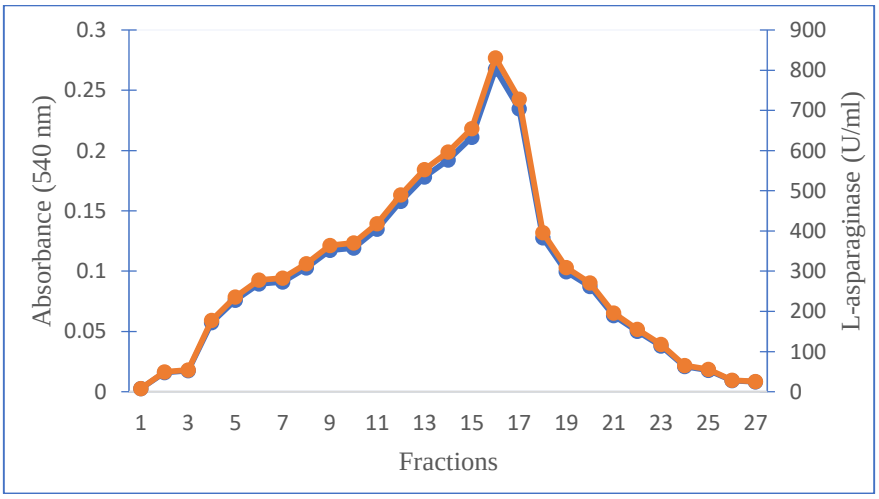


Fig. 2. Elution profile of L-asparaginase

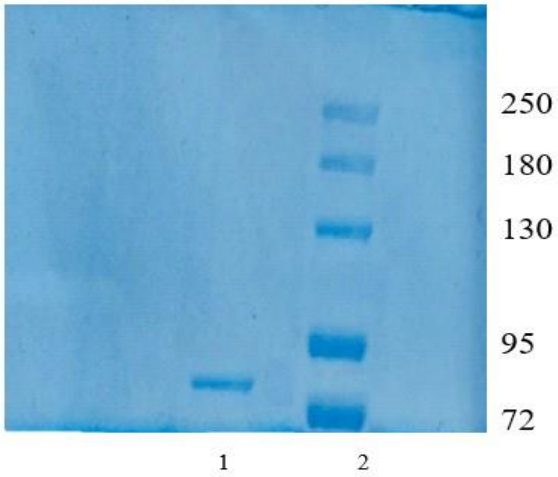
The final phase achieved an overall yield of 50.72% and a L-asparaginase purification fold of 1.68. The purified enzyme's specific activity was found to be 4.70 IU/mg (Table 1).

Table 1. Purification summary of L-asparaginase produced by *S. gilvifuscus* MKA19

Purification Steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	1637.28 ± 1.930	587.22 ± 3.028	2.78 ± 0.115	1± 0.00	100 ± 0.00
Ammonium sulfate precipitation (80%)	1236.03± 2.270	414.30 ± 3.480	2.98 ±0.1	1.07 ± 0.028	75.49 ± 1.745
DEAE Sepharose chromatography	830.43 ± 2.040	176.52 ± 6.050	4.70 ± 0.081	1.68 ± 0.081	50.72± 1.511

Each value is the Mean ± SD of triplicate analysis

The purified enzyme's specific activity was found to be 4.70 IU/mg (Table 1). Through SDS-PAGE, the molecular weight of the isolated L-asparaginase was determined as 85 kDa by comparing it to a known protein molecular weight marker (Fig. 3).



Lane 1: Purified L- asparaginase (85kDa); Lane 2: Protein marker (kDa)

Fig. 3. SDS-PAGE profile of purified L-asparaginase

3.4. Total phenolic content of L-asparaginase

The Gallic acid equivalent (GAE), expressed in mg/g of the enzyme, was used to calculate the total phenolic content using the Folin-Ciocalteu technique. The graph in Figure 4 was used to determine the total phenolic content. The standard curve equation was $y = 0.0011x + 0.0464$, with an R^2 of 0.9809. 0.0352 mg/ml has been found to be the total phenolic content (GAE, mg/g) of the L-asparaginase enzyme.

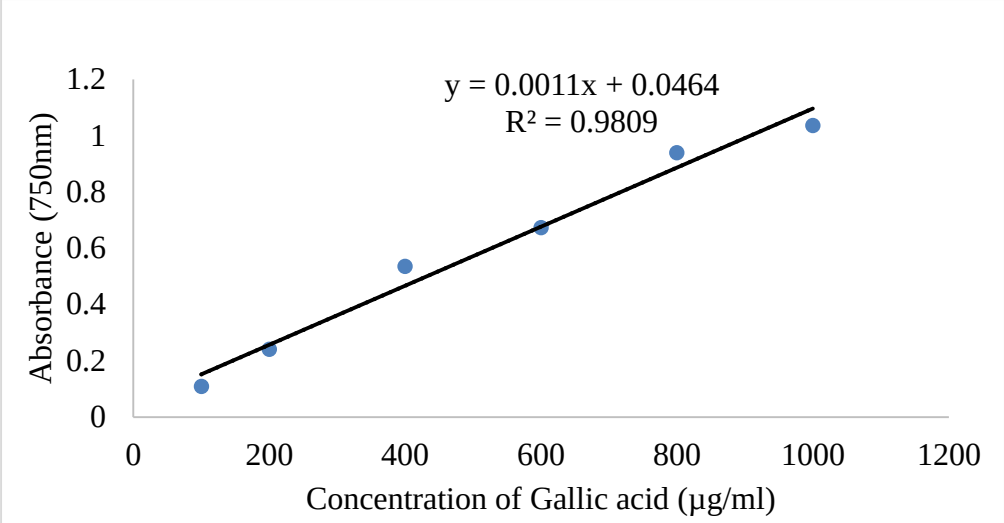


Fig. 4. Total phenolic content of L- asparaginase

3.5. Antagonistic activity of L-asparaginase

Table 2 summarizes the results from the study of the agar-well diffusion method on the antagonistic activity of the isolated enzyme L-asparaginase against human pathogens.

Table 2. Antibacterial activity of L- asparaginase against human pathogens

S. No.	Pathogens	L- asparaginase concentrations			
		10 µl	20 µl	30 µl	40 µl
		Zone of inhibition (mm)			
1.	*S. mutans*	10	14	15	25
2.	*E. faecalis*	17	19	20	20
3.	*S. aureus*	16	17	21	22
4.	*E. coli*	-	-	13	20
5.	*S. pneumoniae*	18	21	23	25

As a result, the inhibitory zones against *E. faecalis*, *E. coli*, and *S. aureus* ranged from 20 to 22 mm at 40 µl concentration. The results showed that the L-asparaginase of the *S. gilvifuscus* MKA19 exhibited the maximum zone of inhibition (25 mm) against *S. mutans* and *S. pneumoniae* at 40 µl concentration. Concurrently, at the lowest enzyme concentration of 10 µl, the enzyme was comparably inhibited at a minimum level (10 to 18 mm) against every examined organism, except for *E. coli*.

3.6. Anticancer activity of L- asparaginase against the selected cancer cell lines

Using three different human cancer cell lines, the MTT test method was used to examine the impact of pure L-asparaginase's *in vitro* anti-cancer activity on the reduction of cell viability. There was a dose-dependent decrease in the quantity of viable cancer cells when the purified L-asparaginase enzyme was used. Table 3-5 shows that the isolated enzyme at the lowest dose (6.25 µg/ml) inhibited the development of HeLa, MCF7, and HT-29 cell lines by 10.337, 4.594, and 11.817%, respectively. Still, in all studied cell lines, the percentage inhibition of cancer cells grew in tandem with an increase in the enzyme's concentration level. At 100 µg/ml concentration, the results demonstrated a significant ($p<0.05$) inhibition of 53.909, 43.892, and 50.513% against the corresponding HeLa, MCF7,

and HT-29 cell lines (Plate 1-3). The corresponding cell line's IC_{50} values were found to be 86.824, 105.681, and 91.318 µg/ml. At the same time, it was revealed that the IC_{50} values of the corresponding cell lines treated using standard quercetin had been 25.01, 19.11, and 45.15 µg/ml.

Table 3. Anticancer activity of different concentrations of L-asparaginase against human cervical cancer cell line (HeLa)

Concentrations (µg/ml)	Anticancer activity against human cervical cancer cell line (HeLa)			
	L- asparaginase enzyme		Standard (Quercetin)	
	Viability (%)	Inhibition (%)	Viability (%)	Inhibition (%)
6.25	89.662±2.665	10.337±0.220[a]	68.20±2.140	31.80±1.146
12.5	79.082±2.486	20.917±0.843[b]	60.60±2.065	39.40±1.163
25	75.458±2.312	24.542±1.040[c]	52.64±1.248	47.36±1.648
50	60.534±1.846	39.465±1.412[d]	42.46±1.044	57.54±2.081
100	47.090±1.583	53.909±1.844[e]	28.09±0.877	71.91±2.840
IC_{50} (µg/ml)	86.824		25.01	

Each value is the Mean ± SD of triplicate analysis; column means with various superscript letters are significant (One-way ANOVA; P<0.05 and *post hoc* multiple comparison with SNK test at 5 % level)

Table 4. Anticancer activity of different concentrations of L-asparaginase against human breast cancer cell line (MCF-7)

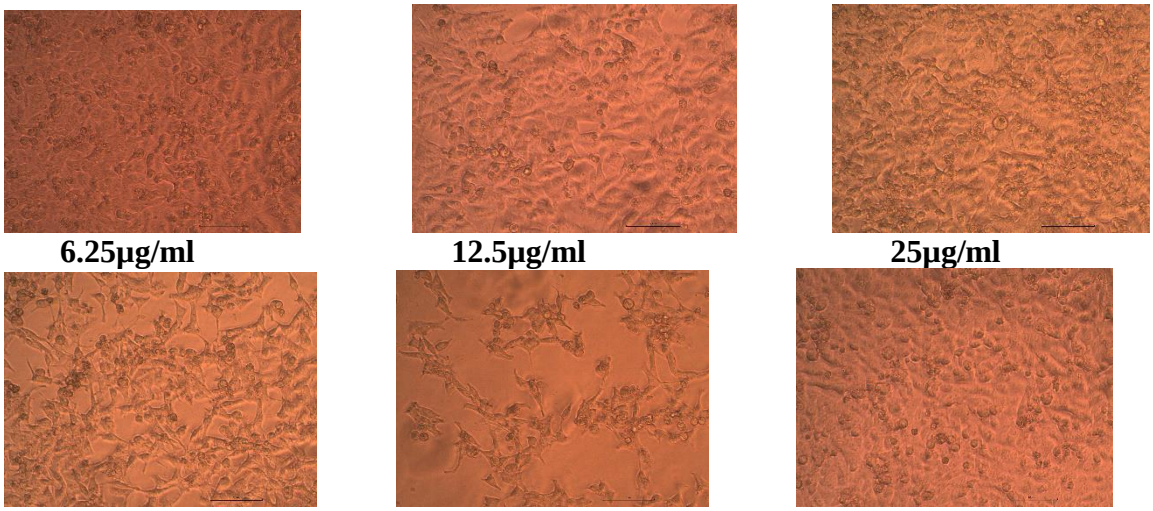
Concentrations (µg/ml)	Anticancer activity against human breast cancer cell line (MCF-7)			
	L- asparaginase enzyme		Standard (Quercetin)	
	Viability (%)	Inhibition (%)	Viability (%)	Inhibition (%)
6.25	95.405±3.354	4.594±0.022[a]	75.25±2.281	20.75±0.882
12.5	80.927±2.743	19.072±0.864[b]	63.06 ±2.048	36.94±1.326
25	71.120±2.263	28.879±1.040[c]	46.31±1.681	53.69±2.173
50	64.145±2.120	35.854±1.412[d]	29.20±0.863	70.80±2.463
100	56.107±1.920	43.892±1.863[e]	5.56±0.148	94.44±3.179
IC_{50} (µg/ml)	105.681		19.11	

Each value is the Mean ± SD of triplicate analysis; column means with various superscript letters are significant (One-way ANOVA; P<0.05 and *post hoc* multiple comparison with SNK test at 5 % level)

Table. 5. Anticancer activity of different concentrations of L-asparaginase against colon cancer cell line (HT-29)

Concentrations (µg/ml)	Anticancer activity against colon cancer cell line (HT-29)			
	L- asparaginase enzyme		Standard (Quercetin)	
	Viability (%)	Inhibition (%)	Viability (%)	Inhibition (%)
6.25	88.182±2.843	11.817±0.860[a]	82.36±2.216	17.64±0.414
12.5	82.365±2.268	17.635±1.002[b]	70.15±2.122	29.85±1.122
25	71.790±1.886	28.209±1.640[c]	61.56±2.048	38.44±1.681
50	61.710±1.465	38.289±1.832[d]	49.77±1.279	50.23±2.089
100	49.487±1.220	50.513±2.012[e]	34.77±1.081	54.85±2.122
IC_{50} (µg/ml)	91.318		45.15	

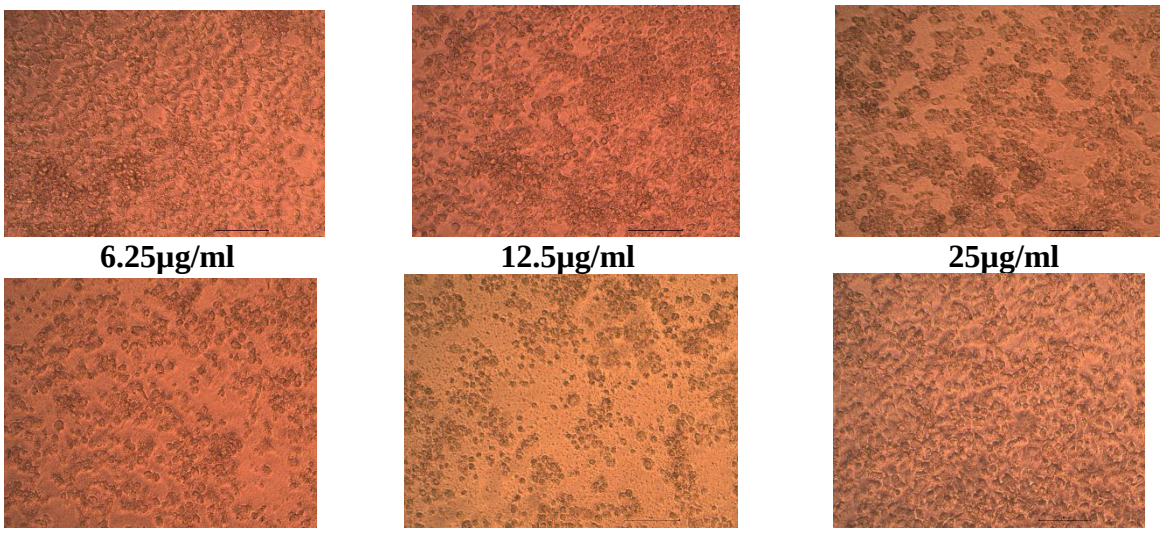
Each value is the Mean ± SD of triplicate analysis; column means with various superscript letters are significant (One-way ANOVA; P<0.05 and *post hoc* multiple comparison with SNK test at 5 % level)



6.25µg/ml **12.5µg/ml** **25µg/ml**

50µg/ml **100µg/ml** **control**

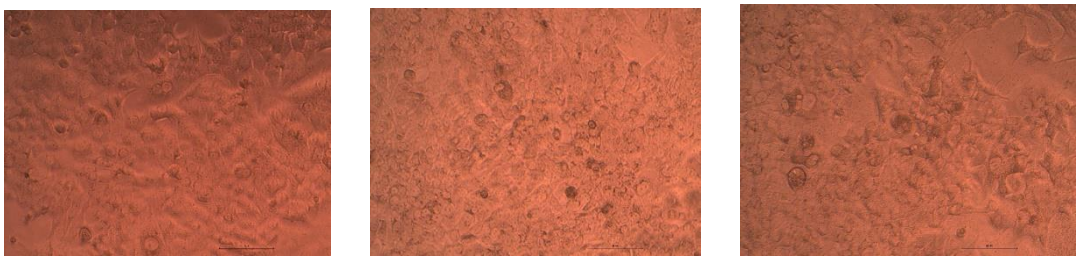
Plate 1. Anticancer activity of different concentrations of L- asparaginase enzyme against human cervical cancer cell line (HeLa)



6.25µg/ml **12.5µg/ml** **25µg/ml**

50µg/ml **100µg/ml** **control**

Plate 2. Anticancer activity of different concentrations of L- asparaginase enzyme against human colon cancer cell line (HT-29)



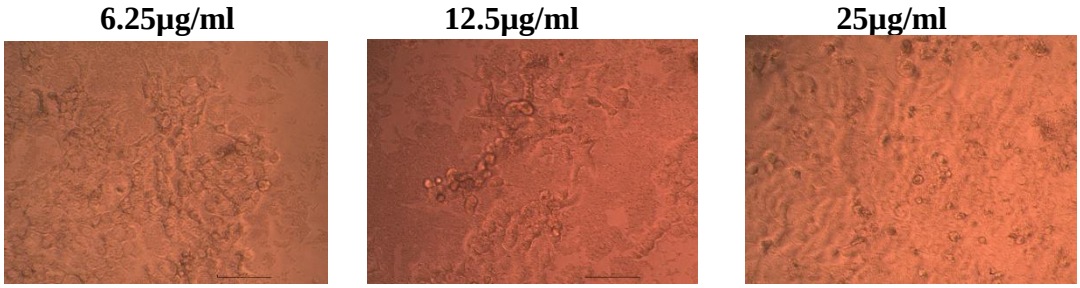


Plate 3. Anticancer activity of different concentrations of L- asparaginase enzyme against human breast cancer cell line (MCF-7)

3.7 Antioxidant Activity of L-asparaginase

Three different assays, including DPPH scavenging, hydroxyl radical scavenging, and nitric oxide scavenging assays, were used to investigate the antioxidant capacity of the isolated L-asparaginase enzyme of *S. gilvifuscus* MKA 19. While the enzyme had been applied at various concentrations for the DPPH scavenging assay (1.25, 2.5, 5, 10, and 20 µg/ml), it was used at 12.5, 25, 50, 100, and 200 µg/ml for the nitric oxide scavenging and hydroxyl radical scavenging activities.

3.7.1 DPPH scavenging activity

At the maximum dose of 20 µg/ml, crude L-asparaginase exhibited better inhibitory activity (30.33%) in terms of its DPPH radical scavenging activity. In this case, increasing L-asparaginase concentrations enhanced the DPPH radical scavenging capacity (Fig. 5). The least amount of DPPH radical scavenging inhibitory activity (2.16%) could be observed at the lowest concentration (1.25 µg/ml). L-asparaginase's DPPH radical scavenging activity has been determined to have an IC_{50} value of 33.66 µg/ml.

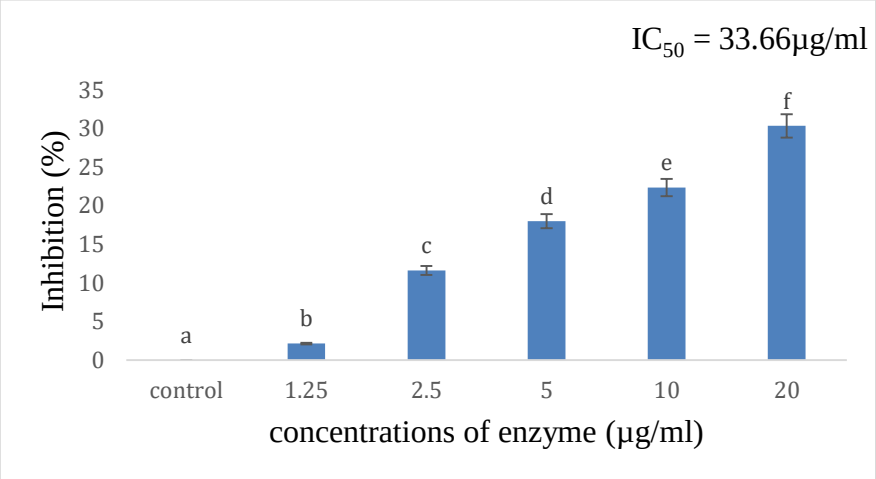


Fig. 5. DPPH scavenging activity of L- asparaginase

Each value is the Mean ± SD of triplicate analysis, bars represented with various superscript letters are significant (One-way ANOVA; $P<0.05$ and *post hoc* multiple comparison with SNK test at 5 % level)

3.7.2 Hydroxyl radical scavenging activity

With increasing doses, the tested L-asparaginase was found to have an increasing in-vitro hydroxyl radical scavenging action. The hydroxyl radical scavenging activity was 7.78% at the enzyme's lowest concentration of 12.5 µg/ml. But the hydroxyl radical scavenging activity showed 37.72% when the concentration level increased to 200 µg/ml (Fig. 6). L-asparaginase's inhibitory concentration (IC_{50}) was found to be 271.67 µg/ml.

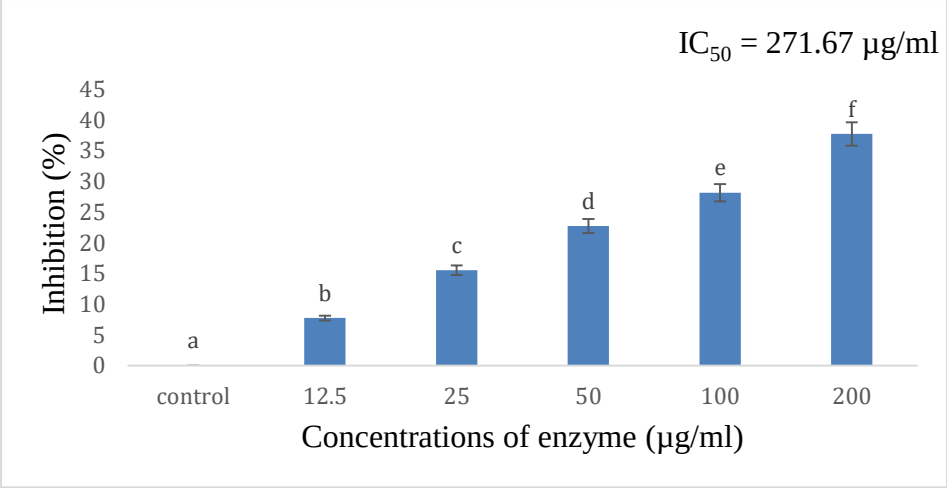


Fig. 6. Hydroxyl radical scavenging activity of L- asparaginase

Each value is the Mean ± SD of triplicate analysis, bars represented with various superscript letters are significant (One-way ANOVA; P<0.05 and *post hoc* multiple comparison with SNK test at 5 % level)

3.7.3 Nitric Oxide scavenging activity

According to Figure 7, there was a noticeable rise in the Nitric oxide scavenging activity of L-asparaginase as concentrations rose. A 4.27% nitric oxide scavenging activity was observed at the lowest enzyme concentration of 12.5 µg/ml. However, the nitric oxide scavenging activity improved to 41.54% at the maximum dose of 200 µg/ml. 221.31 µg/ml was found to be the L-asparaginase IC_{50} value.

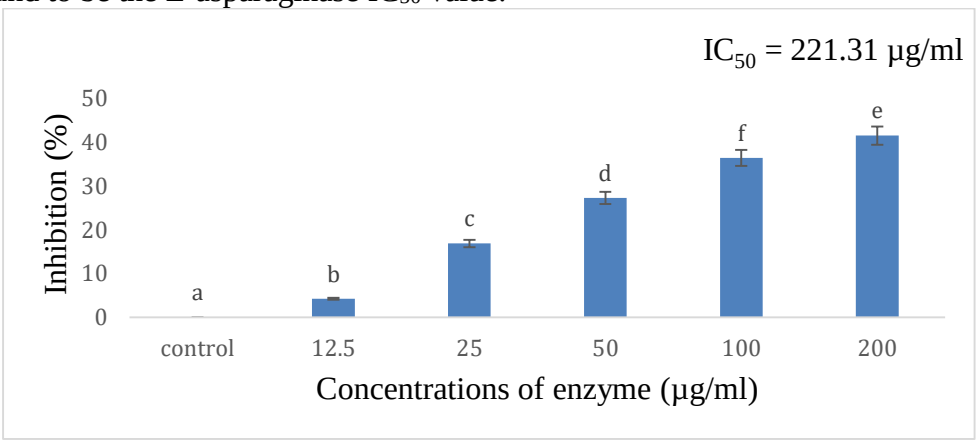


Fig. 7. Nitric oxide scavenging activity of L- asparaginase

Each value is the Mean ± SD of triplicate analysis, bars represented with various superscript letters are significant (One-way ANOVA; P<0.05 and *post hoc* multiple comparison with SNK test at 5 % level)

4. DISCUSSION

Actinomycetes are well-known microbes for their ability to produce antibiotics, herbicides, pesticides and enzymes including asparaginase (Prapagdee *et al*., 2008; Boroujeni *et al*., 2012). As a chemotherapeutic agent, L-asparaginase is a vital enzyme having a wide range of medicinal applications in treatment of infectious diseases, autoimmune diseases, antimicrobial property, etc. Though least explored, the microbes from the marine sources capable of producing asparaginases are expected to possess unique properties like tolerance to various environmental conditions like temperature, pH and salinity. Hence, marine bacteria could be considered as an ideal source for the production of anticancer asparaginases through

submerged fermentation having unique characteristics. In comparison with bacterial and fungal resources, Genus *Streptomyces* is found to be a promising source of L-asparaginase (Sahu *et al*., 2007). The L-asparaginase enzyme produced by *Streptomyces* species from the Bay of Bengal possess good antimicrobial property (Bhargavi and Jayamadhuri, 2016).

In this study, the candidate strain *S. gilvifuscus* MKA19 was previously isolated from the sediment samples of mangrove environment. The L-asparaginase production capacity of this isolate was determined by screening study using L- asparagine with phenol-red indicator, after incubation were observed for pink zone around the colonies and these colonies were taken for further studies. Similarly, Usha *et al*. (2011) enumerated 63 isolates from sediment samples collected from Pichavaram mangrove ecosystem, Tamil Nadu, India. The collected isolates were further screened for L-asparaginase production by means of Rapid plate assay using M-9 medium and testified only 24 isolates were positive for L-asparaginase production. Likewise, Kamble *et al.* (2012) screened 22 actinobacterial strains isolated from farm, water and saline soil samples collected from Virudhunagar region for L-asparaginase production through Rapid plate assay and found only 6 strains were positive for L-asparaginase production. Dhevagi and Poorani (2006) revealed that the strain *Streptomyces sp.* screened for the production of L-asparaginase by rapid plate assay method has the maximum enzyme activity at the rate of 374.6 IU/ml.

In the present study, the ammonium sulphate precipitation and anion exchange chromatography techniques were done for the purification of L-asparaginase enzyme. As a result of final step purification, the specific activity of L-asparaginase was increased from 2.78 to 4.70 IU/mg and the purification fold was also increased from 1.0 to 1.68. The L-asparaginase yield was recorded as 50.72%. The purified L-asparaginase by *S. gilvifuscus* through SDS-PAGE was confirmed to be a single monomeric protein molecule with molecular weight of 85 kDa. Similarly, Desai *et al.* (2016) revealed that L-asparaginase produced by a *Streptomyces sp.* on purification showed a single major band with molecular mass of ≈50 kDa with 3.25% yield. In a study by Silva Lacerda *et al.* (2017), the molecular weight of the purified L-asparaginase from *S. ansochromogenes* was determined as 63.99 kDa with 46% yield. Similarly, Zheng *et al*. (2016) in a study testified that the L-asparaginase from *S. ansochromogenes* showed 14.73% yield and 3.158-fold purification factor.

Current study revealed that the antagonistic activity of L- asparaginase enzyme produced by *S. gilvifuscus* MKA19 could strongly inhibit the growth of human clinical pathogens. For instance, it displayed a maximum growth inhibitory activity against *S. mutans* (25 mm), *S. pneumoniae* (25 mm) and *S. aureus* (22 mm) at 40 µl concentration, whereas the activity was minimum (13 mm) against *E. coli* at 30 µl concentration. This result clearly indicated that the L- asparaginase exhibited strong antagonistic activity and thereby inhibited the growth of various human clinical pathogens. Similarly, Meganathan (2016) has evaluated the antimicrobial activity of L-asparaginase from *Bacillus flexus* RM1 strain, where the maximum antibacterial activity was recorded against *S. aureus* and *P. aeruginosa* at 26 mm, followed by inhibitory activity against *Shigella flexneri* (22 mm) and *Salmonella Typhi* (20 mm). Similarly, antifungal and antibacterial activity of L-asparaginase determined by Raj and Sathiyamurthy (2016) anticipated its significance for therapeutic application against pathogenic microbes. In another study, Vimal and Kumar (2017) identified *Purpureocillium lilacinum*, as higher L-asparaginase producer strain and evaluated its antimicrobial potency against a wide range of Gram-positive/Gram-negative bacteria like *Listeria monocytogenes, Staphylococcus aureus*, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium* and *Proteus vulgaris*.

L-asparaginase are potential enzymes, which could control the growth of cancer cells. Amer *et al*. (2021) assessed the anticancer activity of partially purified L-asparaginase enzyme of strain *Weissella paramesenteroides* isolated from buffalo's colostrum after 48 hrs

of delivery and found that the lowest IC_{50} value of 0.135 mg/ml and consequently the highest SI value of 2.23, when the enzyme was tested against Caco-2 (colon carcinoma cell line). In this study, the L- asparaginase enzyme from the potent actinobacterial strain *S. gilvifuscus* MKA19 possessed the maximum percentage inhibition of cancer cells (43.89 to 53.90%) with the IC_{50} values of 105.681, 91.318 and 86.824 µg/ml against MCF-7 (Human breast cancer cell line), HT-29 (Human colorectal adenocarcinoma cell line) and HeLa (Cervical cell line) respectively. Mahajan *et al*. (2014) and Arjun *et al*. (2016) found that the L-asparaginase produced by marine *Bacillus sp.* and *B. licheniformis* strains possess maximum cytotoxic effect with IC_{50} values of 0.22, 0.78 and 0.153 IU against the human cancer cell lines, viz. Jurkat clone E6-1, MCF-7 and K-562, respectively. Farag *et al*. (2015) reported that the enzyme L-asparaginase produced by *Halomonas elongata* exhibited inhibitory effect against human leukaemia cell lines with the IC_{50} values of 1-2 U/ml. Moreover, L-asparaginase obtained from *Bacillus sp* R36 inhibited the growth of colorectal carcinoma cell line (HCT-116) and hepatocellular carcinoma cell line (HepG2), at IC_{50} values of 218 and 112.19 µg/ml, respectively (Maysa *et al*. 2010). In addition, a study by Shafei *et al*. (2015) described that the L-asparaginase inhibited the growth of breast, hepatocellular and prostate carcinoma cell lines at the IC_{50} values in between 12.5 and 37 µg/ml. Bhagat *et al*. (2016) observed that the L-asparaginase from *Pseudomonas oryzihabitans* after purification showed cytotoxicity effect against MCF-7 at the IC_{50} value of 43.30 µg/ml and HeLa cell lines with the IC_{50} value of 50 µg/ml. Estefania *et al*. (2019) stated that the L-asparaginase produced by *S. lacticiproducens* (strain112) after purification showed the cytotoxic effect on THP-1 (Human leukaemia monocytic cell line) with the IC_{50} value of 36.74 µg/ml. Pokrovskaya *et al*. (2012) in their study testified that the recombinant L-asparaginase enzyme produced by *Yersinia pseudotuberculosis* exhibited the cytotoxicity effect on MDA- MB231 (human breast cancer cell line) with the IC_{50} value of 10 U/ml.

The reduction of DPPH absorption indicated the capacity of a sample to scavenge free radicals, irrespective of any enzymatic activity. With the increase in concentration of the enzyme L-asparaginase, excellent DPPH radical scavenging activity could be achieved (El-Gendy *et al*., 2021). In the present study, the L- asparaginase enzyme of *S. gilvifuscus* MKA19 exhibited the maximum (30.33%) DPPH scavenging activity at 20 µg/ml concentration, but it showed the maximum of 37.72% hydroxyl radical scavenging effect and 41.54% nitric oxide scavenging activity at 200 µg/ml concentrations respectively. Similarly, Rani *et al*. (2011) reported that the enzyme L- asparaginase of a fungus strain *Aspergillus flavus* (KUFS20) isolated from garden soil displayed good antioxidant properties with the IC_{50} value of 263.63 µg/ml by DPPH scavenging method. Another study, by El Gendy *et al*. (2021) demonstrated that L- asparaginase produced by *Fusarium equiseti* AHMF4 exhibited the antioxidant activity on DPPH with the IC_{50} value of 69.12 µg/ml. Similarly, Maysa *et al*. (2010) reported that, partially purified asparaginase produced by the strain *Bacillus sp* R36 showed the antioxidant DPPH activity with the IC_{50} value of 325.40 µg/ml. In the present study, the L- asparaginase from the potent actinobacterial strain *S. gilvifuscus* MKA19 possessed the maximum percentage of inhibition of antioxidant activity with the IC_{50} values of 33.66, 271.67 and 221.31 µg/ml against DPPH scavenging, hydroxyl radical scavenging and nitric oxide scavenging assays, respectively. In accordance with the present study, Saravanakumar *et al*. (2014) revealed that the soil actinobacterium *S. lavendulae* (SCA 5) exhibited good antioxidant properties by DPPH (IC_{50}: 507.61 µg/ml), hydroxyl radical (IC_{50}: 617.84 µg/ml), nitric oxide (IC_{50}: 730.92 µg/ml) and superoxide anion radicals (IC_{50}: 838.83 µg/ml) scavenging activities. The current study thus demonstrated that the enzyme L-asparaginase from the isolated actinobacterial strain, *S. gilvifuscus* MKA19 has antagonistic properties against human clinical pathogens, also possessed both anticancer and antioxidant properties.

5. CONCLUSION

The findings of the present study derived the conclusion that *S. gilvifuscus* MKA19 isolated from the sediment samples of Manakudy Estuary, Kanyakumari District, South India is a potential source for production of L-asparaginase enzyme. Being a well-known chemotherapeutic agent, the isolated L-asparaginase enzyme would receive much attention as it appears to be a potent candidate possessing antagonistic, anticancer and antioxidant properties. However, more studies should be done for large scale production of the enzyme with effective pharmaceutical significance.

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Declarations

Conflict of interest The authors declared that there is no conflict of interest in this research.

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