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# Isolation and Identification of Pigments from Marine Actinomycetes, Along with Their Potential Applications

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***Abstract***

*Natural dyes produced by marine microorganisms particularly actinomycetes—have gained significance as a source of active chemicals and pharmaceuticals with potential for medicinal purposes in recent years. This significant finding served as the foundation for the investigation, which aimed to determine the cytotoxic, antibacterial, and antioxidant properties of a methanolic pigment extract obtained from the marine actinomycetes VES 01 and VES 04. Based on the Brine Shrimp Lethality Test (BSLT), both extracts showed substantial toxicity activity. The actinomycetes VES 01 and VES 04 pigment crude extracts had lethal concentration 50% (LC50) values of 92.64µg/mL and 134.21 mg/mL, respectively, according to the data. When those extracts were evaluated for antibacterial efficacy against a variety of microorganisms, Bacillus subtilis and Escherichia coli showed the best inhibition rates. Additionally, we used 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals to measure the antioxidant activity. The findings demonstrated that the actinomycetes VES 01 and VES 04 pigment crude extracts had inhibitory concentration 50% (IC50) values of 228.08 µg/mL and 346.3 µg/mL, respectively. We identified the chemical components of actinomycete pigment crude extracts using GC-MS analysis. The results showed that the most prevalent chemicals were 5-Methoxypyrrolidin-2-one (30.23%) for VES 04, 1H-Purine-2,6-dione, and 3,7-dihydro-1,3,7-trimethyl- (CAS) (44.65%) for VES 01 and VES 04. This work showed the antibacterial, antioxidant, and toxicological properties of two pigment crude extracts obtained from actinimycetes actinomycetes VES 01 and VES 04. In terms of antibacterial activity against E. coli ATCC 8739, actnomycete VES 04 pigment crude extract was the most effective, whereas actnomycete VES 01 pigment crude extract revealed the highest level of antioxidant activity.*

**Keywords:** *Antioxidant, Actinomycetes, Pigments*

## 1. Introduction

Numerous issues in the pharmaceutical sector have come up in recent years and established worldwide attention. Among these is resistance to various pathogen types. microbes and an increase in death from infections with neurological conditions (Prestinaci et al., 2015; Kharirie and 2020 Andriani). According to Lounou et al. (2017), resistance is the result of persistent alterations by bacteria, viruses, fungi, and parasites that reduce the efficacy of commonly used antimicrobial agents. In addition, difficult-to-treat infections raise the chance of disease severity, mortality, and spread—all of which have long-term effects (Roope et al. 2019). Prolonged infections with bacteria, viruses, or fungi may be the cause of autoimmune diseases and contribute to degenerative conditions. As a result, both now and in the future, the development of novel antimicrobial compounds should be taken into consideration. Degenerative conditions are brought on by aging-related cell damage or other variables influencing the body's free radical count (Sahardi and Makpol 2019). A number of diseases, including Alzheimer's, Parkinson's, atherosclerosis, cardiovascular disorders, hypertension, and type 2 diabetes, are brought on by the buildup of free radicals (Stambler 2017). Naturally, the body's production of antioxidant compounds allows it to reduce free radicals to some extent. The toxic compound is the other bioactive substance that has the potential to protect against degenerative conditions. It has been demonstrated that in certain solid human tumours, the toxic compound tested using the brine shrimp lethality test (BSLT) has a strong correlation with cytotoxic activity (Mclaughlin et al. 1998). Gram-positive filamentous aerobic

bacteria with a high G+C content is known as actinomycetes (Gong et al. 2018; Bhakyashree and Kannabiran 2018). Extensive research on actinomycetes isolated from terrestrial environments resulted in the reported re-discover of natural products. However, according to Liao et al. (2016) and Pudi et al. (2016), marine actinomycetes are essentially a new source of secondary metabolites, such as terpenes, peptides, sterols, fatty acids, alkaloids, amino acids, and their derivatives. Because of its enormous biodiversity and various environmental conditions, the marine environment can produce all of these compounds (Bibi et al. 2020). According to Hifnawy et al. (2020), actinomycetes associated with marine sponges, like Micromonospora sp., have been applied recently to generate a variety of bioactive compounds with unique molecular scaffolds and significant pharmacological activities. Therefore, one of the genera that produces a lot of secondary metabolites is Micromonospora, especially when it comes to bioactive compounds. The Micromonospora genus belongs to the Micromonosporaceae family, which also consists of 32 other genera. According to Trujillo et al. (2015) and Hifnawy et al. (2020), this genus can be found in a variety of geographic environments, which includes soils, mangrove sediments, plants, marine habitats, and extreme environments. It has been determined that over 740 strains of Micromonospora produce a variety of bioactive compounds, one of which is derived from the pigment (Berdy 2005). Carotenoid pigments, which can be yellow, orange, red, purple, brown, or black, are present in *micromonospora* colonies (Sineva et al. 2021). *Micromonospora chalcea* (yellow), M. halophytica (red-brown), M. olivasterospora (olive-green), *M. purpureochromogenes* (dark-brown), and *M. rosaria* (wine-red) are several species that have soluble pigments (Genilloud 2015). This study aimed to test the antimicrobial, antioxidant, and toxicity abilities of the pigment methanol extracts produced by the marine actinomycetes VES 01 and VES 04 in order to determine the potency of pigment crude extract.

## 2. Materials And Methods

Soil samples were collected from five regions of the mangrove ecosystem in Vellar estuary, Tamil Nadu, India (Lat.11°29'N; Long.79°46'E). After taking soil samples at a depth of 5 to 10 cm, the samples were placed in plastic bags and dried right away. After seven days of air drying at room temperature (27±1°C), the soil samples were crushed with a mortar and pestle. For the purpose of isolating the ground soil samples using the soil dilution technique, larger particles like stone and plant debris were removed using a 0.5mm sieve. After that, the debris were kept at 4°C and stored separately in paper bags. Actinomycete VES 01 (greenish-black) and actinomycete VES 04 (orange), two pigmented marine actinomycetes used in this study, were isolated from mangrove soil. Bacillus subtilis ATCC 19659, Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 15442, Staphylococcus aureus ATCC 6538, and Candida albicans ATCC 10231 were the five microbes used in the antimicrobial test.

METHODS

Pigment extraction

The pigment extraction process is based on the methodology slightly modified by Dawoud et al. (2020). Actinomycete cultures were cultivated in 1 L of ISP-4 broth medium, which contained 10 g of soluble starch, 1 g of MgSO4.7H2O, 1 g of NaCl, 1 g of K2HPO4, 2 g of (NH4)2SO4, 2 g of CaCO3, 0.001 g of FeSO4.7H2O, 0.001 g of MnCl2.4H2O, and 0.001 g of ZnSO4.7H2O. The cultures were continuously shaken in an orbital shaker at 100 rpm for 14 days at room temperature. Following that, the actinomycetes cultures were centrifuged for 15 minutes at 6000 rpm. After adding 1 litre of methanol as a solvent, the cell biomass was heated in a water bath at 50°C for 15 minutes, or until the cell turned colourless. Subsequently, the cell biomass and methanol phase were separated through centrifugation at 6000 rpm for 15 minutes. A rotary evaporator was used to evaporate the methanol solvent at 50°C. After being collected and dissolved in 10% dimethyl sulfoxide (DMSO), the crude extracts were kept cold until they could be used in additional laboratory experiments.

The toxicology investigation

Meyer et al. (1982) state that the Brine Shrimp Lethality Test (BSLT) was used for the toxicity test. Each vial held four millilitres of seawater, twenty Artemia salina larvae, and crude extracts of pigment in concentrations of 0, 10, 100, 250, 500, 750, and 1000 µg/mL. For an entire day at room temperature, the vials containing pigment extracts and larvae were incubated under light conditions. After counting the dead larvae, the following formula was used to determine the percentage of mortality:

$$\% \text{ Mortality} = \left(\frac{\sum \text{sample larvae mortality} - \sum \text{Control larvae mortality}}{\sum \text{Total larvae}}\right) \text{X } 100$$

Probit analysis and a linear regression model were employed to calculate each extract's LC50 value.

Antimicrobial test

The disc diffusion method was employed to carry out the antimicrobial activity. A number of clinical isolates were tested against the pigment extracts. The following bacteria were added to a Mueller-Hinton Agar medium: B. subtilis ATCC 19659, E. coli ATCC 8739, P. aeruginosa ATCC 15442, and S. aureus ATCC 6538. In the meantime, C. albicans ATCC 10231 was added to a Potato Dextrose Agar medium. On the surface of the agar plate medium, a paper disc (6 mm) with different extract concentrations (250, 500, 750, and 1000 µg/mL) was placed. The positive control in this experiment was tetracycline at 100 µg/mL, while the negative control was 10% dimethyl sulfoxide (DMSO). Following a 24-hour incubation period at room temperature, the diameter of the inhibition zones that developed on the plates was measured.

Antioxidant test

The DPPH radical was used to measure the antioxidant activity of the pigment extracts according to with Batubara et al. (2009) with minor modifications. 500 µL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent (0.125µM in methanol) was combined with 500 microliters of each extract at different concentrations (0, 7.81, 15.63, 31.25%, 62.5, 125, 250, and 500 µg/mL). A spectrophotometer (Thermo spectronic-Genesis 20, Thermo Fisher Scientific, USA) was used to measure the sample's absorbance after the mixture was left to stand at room temperature for 30 minutes in the dark. A positive control was ascorbic acid. The following formula was used to determine the inhibition percentage

$$\% \text{ Inhibition} = \left[1 - \left(\frac{\text{sample absorbance} - \text{control absorbance}}{\text{Blanko absorbance} - \text{Control absorba}}\right)\right] \text{X}100$$

The IC50 value of every extract was found using a linear regression model.

Chemical composition examination

Gas chromatography was used in the Agilent 5977B GC/MSD (Agilent Technologies, USA) to analyse the pigment extract of the isolates VES 01 and VES 04. An extract solution diluted in methanol, weighing 0.6 µL, was introduced into an HP-5MS column (30 m x 250 µm x 0.25 µm). The oven was set to 80°C at first, and it was raised to 300°C over the course of 20 minutes at a rate of 15°C per minute. The carrier gas utilised was helium gas, flowing at a rate of 1 mL/min. Both the auxiliary temperature and the injection temperature were kept at 300°C. Using the GC-MS Pyrolysis programme (WILLEY9THN 08. L), the result was examined.

## 3. Results

The pigment extraction

On ISP-4 agar medium, the Actinomycete VES 01 and VES 04 showed greenish-black and orange colonies after seven days of room temperature incubation. The VES 01 and VES 04 were cultivated in ISP-4 broth medium and were incubated on a rotary shaker for 14 days before the pigment was extracted. The crude extracts of the pigments showed greenish-black for VES 01 and brownish-orange for VES 04 (Figure 1). Actinomycete VES 01 and VES 04 pigment extract yields were 0.23% and 0.18%, respectively (Table 1).

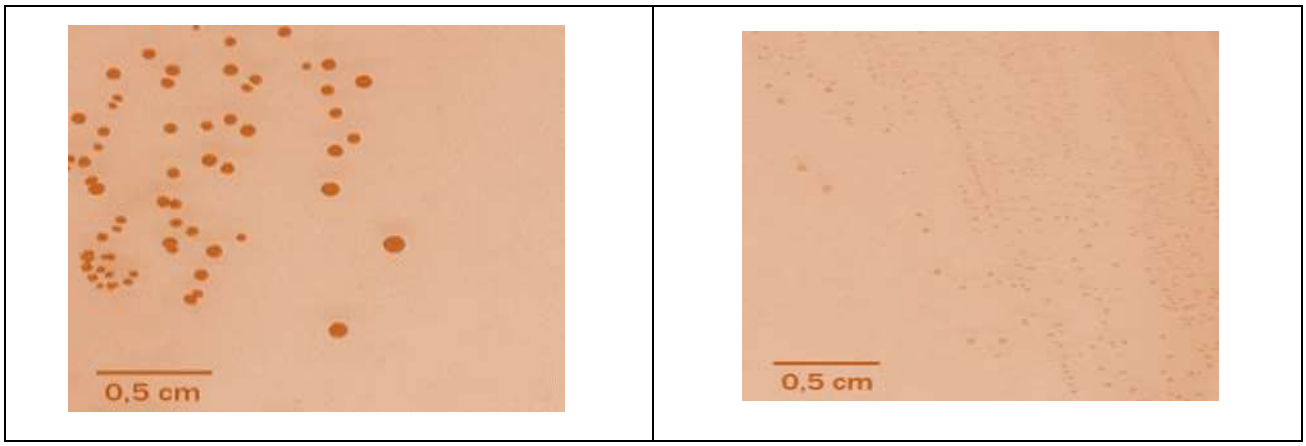


**Figure 1.** The colonies appearance of actinomycetes on ISP-4 agar medium at 7 days of incubation at room temperature and the pigment crude extract yielded after 14 days of culturing in ISP-4 broth medium: A. Actinomycete VES01; B. Actinomycete VES04

**Table 1.** The pigment extract yield derived from Actinomycete VES01 and VES04

| Pigment Extract | Culture Volume (L) | Weight (g) | Yield (%) |
|---|---|---|---|
| Actinomycete VES01 | 1 | 2.33 | 0.23 |
| Actinomycete VES04 | 1 | 1.82 | 0.18 |

Toxicity of pigment extracts

According to the BSLT test, both actinomycete pigment extracts VES 01 and VES 04 were toxic to Artemia salina. The toxicity was measured using the LC50 value. The LC50 value is a standard for the lowest concentration at which 50% of test organisms (A. salina) are executed. The lower the LC50 value, the lower the toxicity. The LC50 values for actinomycete pigment extracts VES 01 and VES 04 were 94.53 g/mL and 131.22 g/mL, respectively (Table 2).

**Table 2.** The toxicity of actinomycete VES01 and VES04 pigment extracts based on Brine Shrimp Lethality Test

| Pigment extract | Replication 1 | Replication 2 | Replication 3 | **Average** |
|---|---|---|---|---|
| Actinomycete VES01 | 88.13 | 80.04 | 115.42 | 94.53 ± 15.14 |
| Actinomycete VES04 | 156.96 | 102.72 | 133.98 | 131.22 ± 22.23 |

Antimicrobial properties of pigment extracts

Actinomycetes pigment extracts were tested against Gram-positive and Gram-negative bacteria, as well as yeast. The pigment extracts of actinomycete VES 01 and VES 04 demonstrated antimicrobial activity against several tested microbes in an antimicrobial test. The VES 01 pigment extract was effective against B. subtilis, E. coli, and S. aureus. VES 04 pigment extract, on the other hand, could inhibit the activity of B. subtilis, E. coli, and P. aeruginosa (Table 3). Both extracts showed no inhibition zone against Candida albicans.

**Table 3.** The antimicrobial activities of actinomycete VES01 and VES04 pigment extracts (750 µg/mL) against several tested microbes

| **Pigment extract** | **Tested microbes** | | | | |
|---|---|---|---|---|---|
| | ***Bacillus subtilis* ATCC 19659** | ***Escherichia coli* ATCC 8739** | ***Pseudomonas aeruginosa* ATCC 15442** | ***Staphylococcus aureus* ATCC 6538** | ***Candida albicans* ATCC 10231** |
| Actinomycete VES01 | + | + | - | + | - |
| Actinomycete VES04 | + | ++ | + | - | - |

The antioxidant properties of pigment extracts

Actinomycete VES 01 and VES 04pigment extracts both exhibited scavenging activity against DPPH as a free radical. The pigment extracts of Actinomycete VES 01 and VES 04 showed IC50 values of 231.08 µg/mL and 369.30 µg/mL, in that order (Table 4). The lowest concentration that can prevent 50% of the activity of free radicals is known as the inhibitory concentration (IC50).

**Table 4.** The antioxidant activities of actinomycete VES01 and VES04 pigment extracts against DPPH as free radical

| Pigment extract | IC50 value (µg/ mL) | | | |
|---|---|---|---|---|
| | Replication 1 | Replication 2 | Replication 3 | Average |
| Actinomycete VES01 | 243.40 | 224.07 | 225.78 | 231.08 ± 8.74 |
| Actinomycete VES04 | 436.77 | 373.95 | 297.18 | 369.3 ± 57.08 |

Identification of chemical compounds

The dominant extract compound was investigated using Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Actinomycete HV11.P3 pigment extract's main constituent was 1H-Purine-2,6-dione,3,7-dihydro1,3,7-trimethyl- (Figure 2; Table 5), whereas actinomycete SCA54's.5-Methoxypyrrolidin-2-one was the main compound in the P2 pigment extract (Figure 3; Table 6).

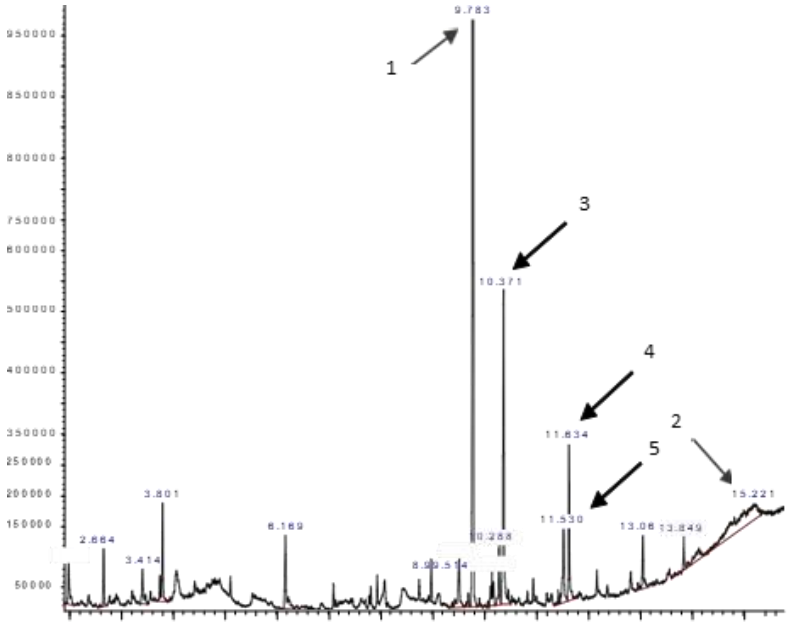


**Figure 2.** Chromatogram of chemical compounds of pigment extract derived from actinomycete VES01 based on GC-MS analysis showing five dominant compounds as indicated by black arrows.

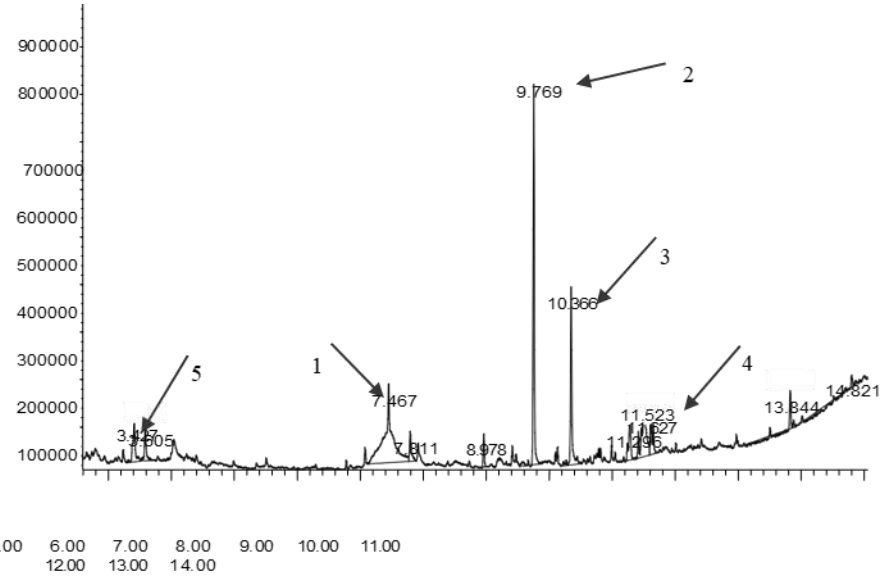


**Figure 3.** Chromatogram of chemical compounds of pigment extract derived from actinomycete VES04 based on GC-MS analysis showing five dominant compounds as indicated by black arrow

**Table 5.** Five major chemical compounds of actinomycete VES01 pigment extract and their biological activities.

| Chemical compound | Peak area (%) | Activity | Reference |
|---|---|---|---|
| 1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl- (CAS) | 44.65 | Anti-inflammation | Rezaei et al. (2021) |
| Cyclotrisiloxane, hexamethyl- (CAS) | 13.20 | Antimicrobial | Keskin et al. (2012) |
| n-Hexadecanoic acid (CAS) | 11.10 | Antibacterial, antioxidant, toxic compound | Krishnaveni et al. (2014) |
| Octadecanoic acid (CAS) | 4.46 | Antibacterial, | Pradheesh et al. (2017) |
| 9-Octadecenoic acid | 3.61 | Anti-hyperglycaemic | Kapoor et al. (2019) |

**Table 6.** Five major chemical compounds of actinomycete VES04 pigment extract and their biological activities.

| Chemical compound | Peak area (%) | Activity | Reference |
|---|---|---|---|
| 5-Methoxypyrrolidin-2-one | 30.23 | Antioxidant | Dascalu et al. (2020) |
| 1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl-(CAS) | 24.27 | Anti-inflammation | Rezaei et al. (2021) |
| n-Hexadecanoic acid | 12.93 | Antibacterial | Krishnaveni et al. (2014) |
| 9,12,15-Octadecadienoic acid (Z,Z,Z)- | 12.26 | Antibacterial | Ojinnaka et al. (2015) |
| 4H-Pyran-4-one, 2-hydroxy-3-methyl-(CAS) | 5.05 | Anti-proliferative, antioxidant | Amatori et al. 2010 |

## Discussion

In our investigation, we discovered that VES 01 and VES 04 could produce orange and greenish-black intracellular pigments, respectively. The BSLT test revealed that the pigment extract of VES 04 and VES 01 had an LC50 value of less than 150 µg/mL, indicating a high in vivo toxicity effect. The BSLT test was employed as a screening tool in the hunt for anticancer drugs (Elsyana et al. 2016). Consequently, more research is required to assess the cytotoxic activity against human cells. Antimicrobial properties that show promise will result in high levels of toxicity (Pandit et al. 2018). It has been discovered that some compounds, like antimicrobial peptide (AMP), have both cytotoxic and antimicrobial properties (Felicio et al. 2017). The actinomycete VES 01 and VES 04 pigment crude extracts exhibited a moderate to low level of activity against the bacteria that were tested. The Gram-positive and Gram-negative bacteria that were tested could both be inhibited by the extracts. As a representative of the eukaryotic cell, C. albicans, was not active against any of these extracts. Actinomycetes are the source of pigments that have attracted a lot of interest because of their potential therapeutic uses, including antimicrobial activity (Mumtaz et al. 2018). Both of our pigment extracts were evaluated for their in vitro antioxidant activities in order to look into their antioxidant activity. Actinomycete VES 01 and VES 04 pigment extracts had IC50 values of 23.08 g/mL and 369.30 g/mL against DPPH radicals, respectively. The antioxidant may reduce the level of oxidative stress in the cell. The low level of free radicals undoubtedly reduces the possibility of cell damage and the efficacy of antibiotics. Some antioxidants, on the other hand, have been shown to have potent antibacterial activity. The mechanisms involved in this process have previously been described (Naqvi et al. 2019; Dwyer et al. 2014). It's interesting to note that both of our crude extracts had antibacterial and antioxidant properties. Our findings were consistent with previous research using Streptomyces sp. VITSTK7, which had both antibacterial and antioxidant properties (Thenmozhi and Kannabiran 2012). GC-MS analysis was also used to determine the chemical composition of the pigment extracts in order to better understand the possible chemical compounds involved in their biological activities. Benzene, alcohols, esters, fatty acids, and amino acid groups are among those compounds. In our current study, the presence of n-Hexadecenoic acid (CAS), Octadecanoic acid (CAS), and 9,12,15-Octadecadienoic acid (Z, Z, Z) may be the main compounds that inhibit microbial growth. Our findings support previous research that found these compounds can inhibit the growth of bacteria like E. coli (Gram-negative bacteria), S. aureus (Gram-positive bacteria), and the fungus Aspergillus flavus (Krishnaveni et al. 2014).

## 4. Conclusion

Meanwhile, n-Hexadecenoic acid (CAS) and 5- Methoxypyrrolidin-2-one, which may act as an antioxidant, may also play important roles (Dascalu et al. 2020). Antibacterial, antioxidant, and toxicity activities were demonstrated by pigments derived from two actinomycetes, Micromonospora chalcea

VES01 and Micromonospora tulbaghiae VES04. M. tulbaghiae VES04 pigment extract demonstrated the best antibacterial activity against E. coli ATCC 8739, while M. chalcea VES01pigment extract demonstrated the best antioxidant activity.

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