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Bioactivity potential and chemical profile of endophytic *Stutzerimonas stutzeri* strain D2 isolated from *Myristica fatua* Houtt

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ABSTRACT: Endophytic bacteria isolated from medicinal plants are recognized as a source of valuable secondary metabolites. The present study aimed to isolate and evaluate the potential antibacterial, antioxidant, and cytotoxic properties of endophytic bacteria associated with *Myristica fatua* Houtt, along with molecular identification and chemical composition analysis of the most potential isolate. A total of 25 isolates were obtained from the root, leaf, and stem bark of *M. fatua*. 16 isolates (64%) exhibited antibacterial activity against at least one target strain. Preliminary screening showed that the D2 isolate displayed remarkable antibacterial activity against four targeted strains. D2-derived metabolite extract possessed moderate antibacterial activity with minimum inhibitory concentration (MIC) of 1,875 µg/mL for *E. coli, P. aeruginosa*, and *S. aureus,* and 3,750 µg/mL for *B. subtilis*, and minimum bactericidal concentration (MBC) of > 3,750 µg/mL. Furthermore, D2 extract exhibited weak antioxidant activity against DPPH (IC_{50}: 242.61±3.2 µg/mL) and ABTS (IC_{50}: 317.32±9.3 µg/mL). In the concentration of 100 µg/mL, the extract was able to decrease the viability of MCF-7 and HepG2 cells, with inhibition percentages of 52.6±9.7 and 41.5±9.36%, respectively. 16S rRNA-based identification confirmed D2 isolate was very similar (99.92%) to *Stutzerimonas stutzeri* strain SM12. The total phenolic content (TPC) and total flavonoid content (TFC) of the D2 extract were 21.11±2.3 mg GAE/g extract and 8.12±1.2 mg QE/g extract, respectively. At least fifteen volatile compounds were found in the D2 extract, as identified using GC-MS analysis. Four out of 15 compounds are well known responsible for antimicrobial, antioxidant, and cytotoxic properties, such as 2,4-di-tert-butyl phenol, pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), and bis(2-ethylhexyl) phthalate. The present study suggests that endophytic *S. stutzeri* strain D2 isolated from *M. fatua* Houtt could be a good source of active compounds valuable for pharmaceutical purposes.

KEYWORDS: Antibacterial; antioxidant; cytotoxic; endophyte; *Stutzerimonas stutzeri*

1. INTRODUCTION

Myristica fatua Houtt. (Ambon forest nutmeg), an evergreen tree belonging to the family of Myristaceae is one of Indonesia's native plants, distributed in Sulawesi, Kalimantan, and Moluccas [1]. This plant also could be found in India, Malaysia, New Guinea, the Philippines, Solomon Island, and The South Pacific Island [2]. The plant has an oblong-ovoid to ellipsoid capsule fruit. The plant could produce essential oil, which could be extracted from the mace, seed, kernel, and leaf [3]. The essential oil isolated from this plant contained sabinene, 4-terpineol, myristicin, eugenol, safrole, and its derivatives [4]. Several studies reported that active compounds derived from this plant have biological activities, such as cytotoxic [5], antioxidant and antibacterial [6], inhibition to α-glucosidase activity [7], analgesic and anti-inflammatory [8]. Therefore, this plant is considered a valuable medicinal plant in Indonesia.

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Medicinal plants are mostly colonized by endophytic microbes (bacteria and fungi). The microbes colonize their host tissue without causing obvious symptoms or making apparent damage to the host [9]. Currently, the isolation and identification of endophytic bacteria have been considered for discovering novel active compounds. Some new compounds were identified produced by endophytic bacteria, such as ecomycine, pseudomycins, munumbicin, kakadumycin, and xiamycin [10]. Diverse endophytic bacteria that exist within medicinal plant tissue also produce many therapeutic compounds that are attributed to antibacterial [11], antioxidant [12], and cytotoxic properties [13]. Hence metabolites synthesized by endophytes increased attention as future prospective antioxidants, antibiotics, and cytotoxic agents.

Even though the beginning of the study on endophytes initiates since the 19th century, there are still many endophytes associated with Indonesia's native plant species is not studied yet, especially endophytic bacteria from *M. fatua*. This study aimed to evaluate the antibacterial, antioxidant, and cytotoxic properties of endophytic bacteria isolated from *M. fatua*. This study has also carried out phytochemical analysis (total phenolic content, total flavonoid content, and GC-MS profile) and molecular identification of the most potential isolate.

2. RESULTS AND DISCUSSION

2.1. Endophytic bacteria isolated from *M. fatua* and their antibacterial activity

A total of 25 isolates with different colony morphologies was obtained from *M. fatua* plant parts, of which 60% (15 isolates) were isolated from the root, 8% (2 isolates) from the leaf, and 32% (8 isolates) from the stem bark (Table 1). More bacterial endophyte isolates found in roots indicate that roots provide a more favorable environment for bacterial colonization. Earlier studies also reported that the number of endophytic bacteria in roots of *Leucojum aestivum* [14], *Lactuca indica* [15], and *Targetes* sp. [16], was higher than aerial plant parts. It might be influenced by the fact that the root endophytic bacteria might be originated from the soil, one of the the most favorable environment of microbial community. Endophytic bacteria could enter the plant tissue through roots, exist within the plant tissue host, and then spread systematically inside the plant organ [17]. Interestingly, in this study, nearly 64% (16 isolates) exhibited antibacterial activity against four target strains (*Escherichia coli* strain ATCC 8739, *Pseudomonas aeruginosa* strain ATCC 15442, *Bacillus subtilis* strain ATCC 19659, and *Staphylococcus aureus* strain ATCC 6538) with different antibacterial spectra. These isolates inhibited at least one target strain, indicated by forming a clear zone around the endophytic bacterial colony. Among other isolated bacteria, the isolate coded as D2 showed broad antibacterial activity against all the target strains (Table 1).

The antibacterial property of endophytic bacteria is commonly promoted by the ability of the bacteria to synthesize antibacterial compounds. Metabolites extract derived from the most potential isolate, D2 isolate, was further investigated for its antibacterial activity. The results showed that the extract displayed antibacterial activity at 10 mg/mL concentrations against four target strains (Table 2, and Figure 1). In line with the result of the initial antibacterial screening through the dual culture test, the extract was also most active in *B. subtilis* and *P. aeruginosa* than in other target bacteria. Each target bacteria have different susceptibility to D2 extract as it might be influenced by their resistance capability and external cell structure. Furthermore, the MIC (the lowest concentration of antibacterial substances that inhibit the visible growth of certain bacteria) and MBC (the lowest antibacterial compound concentration that kills 99.9% of bacteria) of the D2 extract have also been investigated in this study (Table 3). D2 extract exhibited MIC 1,875 µg/mL for *E. coli, P. aeruginosa*, and *S. aureus* and 3,750 µg/mL for *B. subtilis*. In these MIC concentration ranges, four target strains were susceptible (0.001<MIC<0.5 mg/L) to D2 extract [18]. Whereas the extract has MBC higher than 3,750 µg/mL, meaning that a higher concentration of this extract is still needed to kill the target strains completely. The lower MIC and MBC concentration were shown by tetracycline as a positive control. Similarly, MIC and MBC values range were also performed by the host plant extract. Aril and Kernel of *M. fatua* extracts have a MIC range of 1,250 – 10,000 µg/mL and a MBC range of 5,000-10,000 µg/mL against *B. subtilis* and *S. aureus* [6].

Table 1. The antibacterial activity of endophytic bacteria by dual culture method

No	Isolate Code	Plant organ	İnhibition zone (mm)*			
			E. coli ATCC 8739	*P. aeruginosa* ATCC 15442	*B. subtilis* ATCC 19659	*S. aureus* ATCC 6538
1	A1	Root	-	+	-	+
2	A2	Root	-	-	-	-
3	A3	Root	-	-	-	-
4	A4	Root	-	-	-	-
5	A5	Root	-	+	-	+
6	A6	Root	-	-	-	-
7	A7	Root	-	-	-	-
8	A8	Root	-	+	-	-
9	A9	Root	-	+	-	-
10	A10	Root	-	+	-	-
11	A11	Root	+	-	-	-
12	A12	Root	+	-	-	-
13	A13	Root	+	-	-	+
14	A14	Root	+	-	-	-
15	A15	Root	++	+	-	-
16	D1	Leaf	+	-	-	-
17	D2	Leaf	+	++	++	+
18	B1	Stem Bark	-	+	-	-
19	B2	Stem Bark	-	+	-	-
20	B3	Stem Bark	-	-	-	-
21	B4	Stem Bark	-	-	-	-
22	B5	Stem Bark	-	+	-	-
23	B6	Stem Bark	-	-	-	-
24	B7	Stem Bark	-	-	-	-
25	B8	Stem Bark	-	+	-	-

*Note: - =No activity; + = less active (clear zone diameter: ≤7 mm), ++ = moderately active (clear zone diameter: 8-13 mm), +++ = highly active (clear zone diameter: >13 mm),

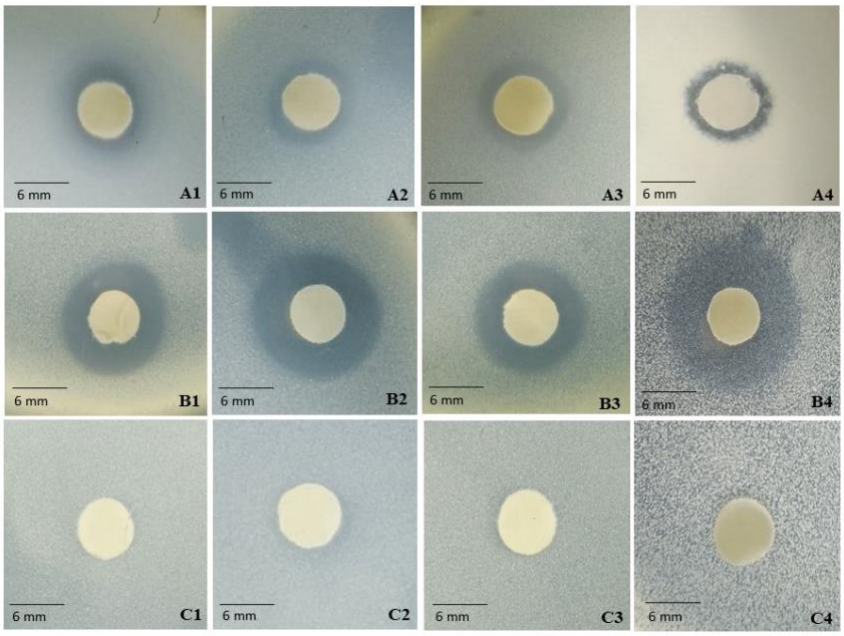

Figure 1. Antibacterial activity of (A1-A4) D2 bacterial endophytic extract (10 mg/mL), (B1-B4) tetracycline (100 µg/mL), (C1-C4) DMSO (1% v/v), against (A1-C1) *Escherichia coli*, (A2-C2) *Pseudomonas aeruginosa*, (A3-C3) *Bacillus subtilis*, (A4-C4) *Staphylococcus aureus* in MHA medium after incubation for 24 h at 37°C.

Table 2. The antibacterial activity of D2 bacterial endophytic extract by disc diffusion method

Sample*	Inhibition zone (mm)			
	E. coli ATCC 8739	*P. aeruginosa* ATCC 15442	*B. subtilis* ATCC 19659	*S. aureus* ATCC 6538
D2 extract	7.8±08	8.1±0.3	8.3±0.5	7.6±0.4
Tetracycline	11.6±0.9	13.1±0.4	12.1±0.6	12.9±0.3
DMSO	0±0	0±0	0±0	0±0

*Note: D2 extract, tetracycline, and DMSO are used at 10 mg/mL, 100 µg/mL, and 1% (v/v), respectively.

Table 3. The MIC and MBC values of D2 bacterial endophytic extract

Sample	MIC and MBC values (µg/mL)							
	E. coli ATCC 8739		*P. aeruginosa* ATCC 15442		*B. subtilis* ATCC 19659		*S. aureus* ATCC 6538	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
D2 extract	1,875	>3,750	1,875	>3,750	3,750	>3,750	1,875	>3,750
Tetracycline	3.91	>7.81	3.91	>7.81	3.91	7.81	3.91	7.81

2.2. Antioxidant and cytotoxic properties of D2 extract

Endophytic bacteria are considered one of the most prospective sources of bioactive compounds attributed to various pharmacological activities. Beside investigating the antibacterial activity of D2 extract, this study also evaluated the antioxidant and cytotoxic properties of D2 extract. D2 extract exhibited antioxidant activity against two free radicals, including DPPH (IC_{50}: 242.61±3.2 µg/mL) and ABTS (IC_{50}: 317.32±9.3 µg/mL) (Table 4). According to its IC_{50} values (>150 µg/mL), D2 extract has weak antioxidant activity [19]. However, the antioxidant of D2 extract (extract derived from leaf endophytic bacteria from *M. fatua*) is still more active than leaf extract of the plant extracted with various organic solvents, which is ranging from 535-2,630 µg/mL [6].

Table 4. Antioxidant activity of D2 bacterial endophytic extract

Sample	Antioxidant activities	
	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
D2 extract	242.61±3.2^{c}	317.32±9.3^{c}
Ascorbic acid	3.71±3.4^{a}	6.39±1.4^{a}
Trolox	5.29±1.4^{b}	16.22±3.3^{b}

In this study, the cytotoxic property of D2 extract was also evaluated on MCF-7 (human breast cancer cell line) and HepG2 (human liver cancer cell line). In the concentration of 100 µg/mL, the extract inhibited the viability of MCF-7 and HepG2 cells, with inhibition percentages 52.6±9.7 and 41.5±9.36%, respectively (Table 5). The results indicate that the extract is moderately active against MCF-7 cells and weakly active against HepG2 cells, according to the categorization of natural product cytotoxicity values [20]. The extract treatment induced morphological changes in cancer cells, including cellular shrinkage, and membrane blebbing and may lead to the apoptosis phase of the cancer cell. Consequently, cancer cell density decreased after treatment (Figure 2). The cytotoxic of D2 extract against MCF-7 cells is still lower than the two purified compounds, including i(7S, 8R, 8'S, 7'S) 7,7'-bis(3-hydroxy-5-methoxyphenyl)-8,8-dimethylbuthane-7,7'-diol, and 3-hydroxydemethyldactyloidin, isolated from *M. fatua* leaves, with an IC_{50} value of 26.19 and 8.33 µM, respectively, against MCF-7 cells [5].

Table 5. Cytotoxicity of D2 bacterial endophytic extract

Sample*	% Inhibition against Cell lines	
	MCF-7 (%)	HepG2 (%)
D2 extract	52.6±9.7^{b}	41.5±9.36^{b}
Cisplatin	89.1±2.8^{c}	82.8±8.1^{c}
DMSO	0±0^{a}	0±0^{a}

*(D2 extract and Cisplatin), and DMSO are used at final concentration of 100 µg/mL and 0.01%, respectively

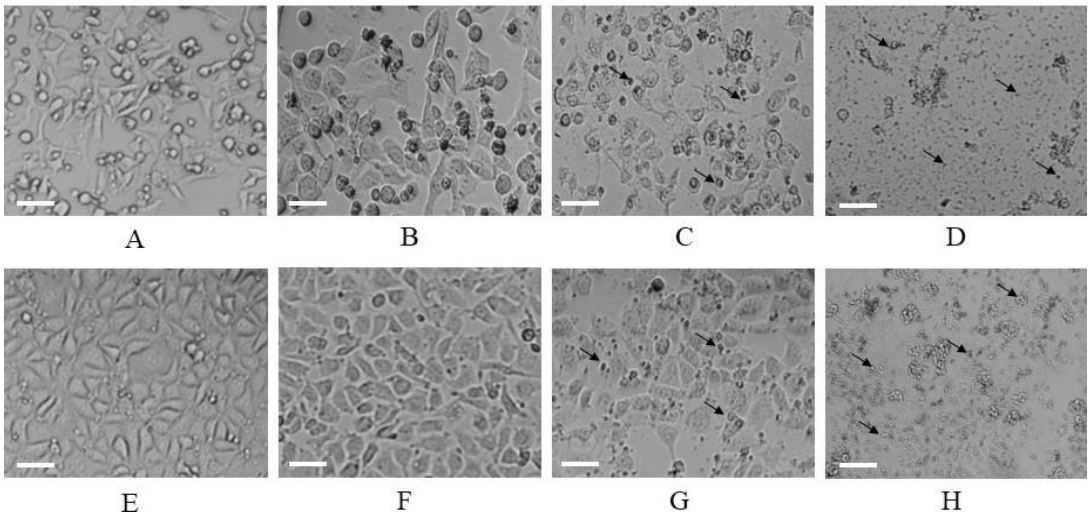

Figure 2. Density and morphology of HepG2 cell line (A-D) and MCF-7 cell line (E-H); HepG2 cell line on (A) DMEM medium following treatment with (B) 1% DMSO, (C) D2 extract 100 µg/mL, and (D) Cisplatin 100 µg/mL; MCF-7 cell line on (E) DMEM medium, and treatment with (F) 1% DMSO, (G) D2 extract 100 µg/mL, and (H) Cisplatin 100 µg/mL. Figures were taken under an inverted microscope with a magnification of 100x. Black arrows show apoptotic cells, and bars represent 30 µm.

2.3. Molecular identity of D2 isolate

Extract derived from D2 isolate, obtained from *M. fatua* leaf, was characterized to have remarkable antibacterial property, and moderate antioxidant and cytotoxic properties. 16S rRNA-based identification showed that this isolate was highly similar (99.92%) to *Stutzerimonas stutzeri* strain SM12 (accession no. MT356167.1), with a query cover of 100%, and an e-value of 0.0. The sequence of D2 isolate has been deposited to NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) under accession number of OQ608627.1. As shown in the phylogenetic tree (Figure 3), the D2 isolate has also belonged to the Stutzerimonas clade. Currently, the Stutzerimonas genus was newly proposed within the *Pseudomonadaceae* family. *Stutzerimonas stutzeri* is the current species name of *Pseudomonas stutzeri* [21]. The species have also been reported as an endophyte of *Withania somnifera*, *Physalis ixocarpa*, *Salicornia europaea*, *Ulva reticulata,* and characterized with biocontrol potential against Fungi (*Fusarium oxysporum*, *Rhizoctonia solani, Botrytis cinerea*) and bacteria (*Bacillus subtilis, Pseudomonas aeruginosa, Salmonella thyphi, Staphylococcus aureus, Escherichia coli*), and plant growth promoting activity under high salinity soil [22-25]. This species could also be found in marine environments and is capable of producing zafrin, a novel antibacterial compound [26].

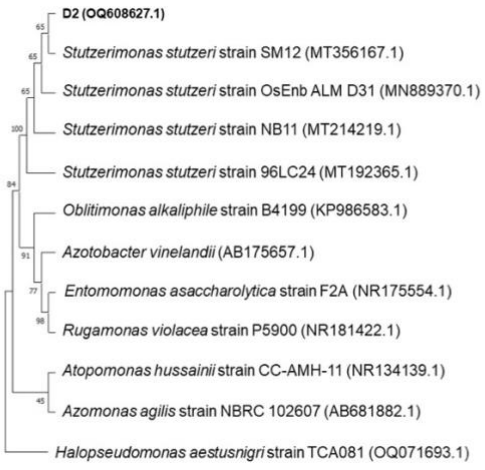

Figure 3. Phylogenetic tree of D2 endophytic bacterial isolate compared to its related strains based on 16S rRNA sequence.

2.4. Phytochemical profile

The chemical profile of the D2 extract was investigated through quantitative (total phenolic content-TPC and total flavonoid content-TFC) and GC-MS analysis. The TPC and TFC of the D2 extract were 21.11±2.3 mg GAE/g extract and 8.12±1.2 mg QE/g extract, respectively. Phenolic and flavonoid were widely studied and responsible for various biological activities of natural products [27-28]. Furthermore, GC-MS analysis showed that D2 extract contained at least 15 volatile compounds with different retention time and relative abundance (Figure 4). Four out of 15 compounds are well known responsible for antimicrobial, antioxidant, and cytotoxic properties, such as 2,4-di-tert-butyl phenol, pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), and bis(2-ethylhexyl) phthalate, and found in other biological sources (Table 6). The biological activity of other 11 compounds found in D2 extract is still not studied yet.

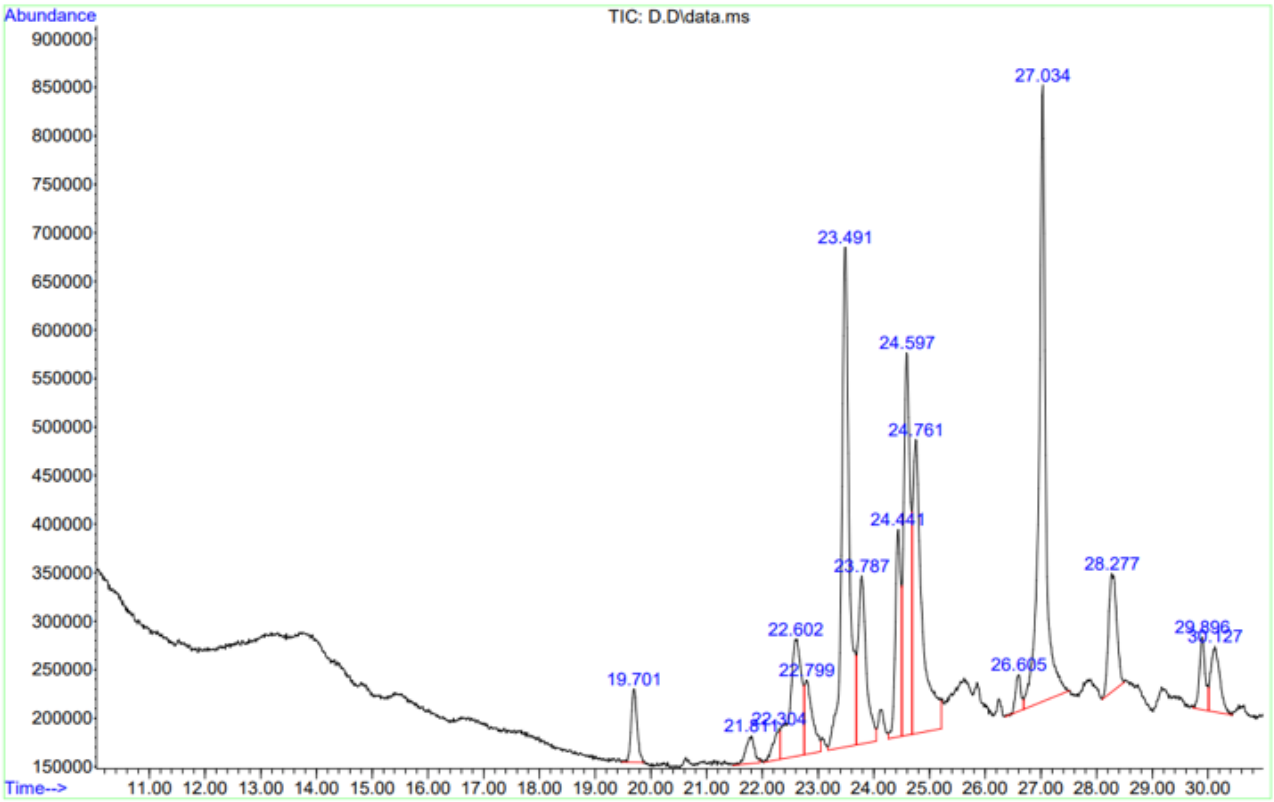

Figure 4. GC-MS chromatogram profile of D2 endophytic bacterial extract

3. CONCLUSION

To our knowledge, this is the first investigations on the antibacterial, antioxidant, and cytotoxic properties of *Stutzerimonas stutzeri* endophytic bacterial strain. The ethyl acetate extract of this endophytic bacterium showed antibacterial activity againts *E. coli, P. aeruginosa*, *S. aureus,* and *B. subtilis* in moderate level. Furthermore, *S. stutzeri* strain D2 extract exhibited antioxidant capacity against both DPPH and ABTS radicals with an IC_{50} value of 242.61±3.2 and 317.32±9.3 µg/mL, respectively. In addition, this extract also possesses cytotoxicity against MCF-7 and HepG2 cells lines with inhibition value of 52.6±9.7 and 41.5±9.36%, respectively. Moreover, the screening of volatile compounds derived from GC-MS analysis of the ethyl acetate extract of *S. stutzeri* strain D2 extract indicated the presence of some compounds that have been previously reported to show potential antimicrobial, antioxidant, and cytotoxic activities, including pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), 2,4-di-tert-butyl phenol, and bis(2-ethylhexyl) phthalate.

Table 6. Compounds present in D2 extract and their biological activity

No.	Compound names	Retention time	Abundance (%)	Similarity (%)	Other sources of compounds	Bioactivities	References
1	2,4-Di-tert-butyl phenol	19.699	2.09	96	*Lactococcus* sp., *Streptomyces* sp. KB1, *Daldinia eschscholtzii*	Antioxidant, antifungal, anti-methicillin resistant *S. aureus* (MRSA), anti-quorum sensing *P. aeruginosa*	[29-31]
2	2H-Azepin-2-one, hexahydro-7-methyl	21.816	1.14	14	-	Unkown	-
3	Acetic acid n-octadecyl ester	22.307	0.95	30	-	Unknown	-
4	1,4-diazabicyclo [4.3.0] nonan-2,5-dione, 3-methyl	22.597	6.82	60	-	Unknown	-
5	6-Dodecene	22.799	2.85	38	-	Unkown	-
6	Cyclo(L-prolyl-L-valine)	23.492	18.21	98	-	Unkown	-
7	L-Proline, N-valeryl-, hexyl ester	23.782	6.68	50	-	Unkown	-
8	1,2-Cyclopentanedione, 3,3,5,5-tetramethyl	24.437	5.34	53	-	Unkown	-
9	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	24.601	11.87	95	*Bacillus tequilensis* MSI45, *Streptomyces mangrovisoli* sp. Nov., *Nocardiopsis* sp. GRG 1, Pseudomonas sp	Anti-multidrug-resistant *S.aureus*, antioxidant, antibiofilm, antiaging	[32-35]
10	Phenol, 3,5-dimethoxy	24.765	13.40	30	-	Unknown	-
11	Octadecanoic acid, ethyl ester	26.605	0.93	95	-	Unknown	-
12	Acetyl tributyl citrate	27.034	20.21	72	-	Unknown	-
13	1-Hexacosene	28.281	4.61	98	-	Unknown	-
14	Bis(2-ethylhexyl) phthalate	29.894	2.03	68	*Lactiplantibacillus plantarum, Clotropis gigantea (Linn.)* flower, octopus (*Paraoctopus* vulgaris)	Antibacterial, larvicidal, antimicrobial, cytotoxic, antimutagenic	[36-38]
15	1H-Indole, 7-methyl-	30.121	2.87	55	-	Unknown	-

4. MATERIALS AND METHODS

4.1. Plant sample collection and bacterial isolates

Healthy disease-free of *Myristica fatua* Houtt. plant was collected from Puspiptek conservation garden, Serpong, South Tangerang City, Banten, Indonesia (GPS: 6°21'12.6"S 106°39'31.2"E). As for bacterial tested belong to the ATCC strains including *Escherichia coli* strain ATCC 8739, *Pseudomonas aeruginosa* strain ATCC 15442, *Bacillus subtilis* strain ATCC 19659, and *Staphylococcus aureus* strain ATCC 6538, kindly provided by Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University. MCF-7 and HepG2 cell lines used in this study are obtained from the collection of Laboratory of Biochemistry, Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research, and Innovation Agency (BRIN), Serpong, Indonesia.

4.2. Isolation of bacterial endophytes

Bacterial endophytes were isolated from the leaf, stem bark, and root of the plant by a method described by Singh *et al.* [39]. In short, surface sterilization of the targeted organ was done by washing with tap water to remove dust and then rinsing with sterile distilled water. Further, the washing was continued with 70% ethanol for 1 min and 0.5% sodium hypochlorite (NaOCl) for 3 min, followed by 96% ethanol for 30 s. The rinsing with sterile distilled water on the final wash was spread on Nutrient Agar (NA) agar plates to determine the success of the surface sterilization method. Serial dilutions of up to 10^{-3} were prepared and 0.1 mL of the dilutions were spread on NA agar plates supplementing with 100 µg/mL Nystatin antifungal (Sigma) in triplicates and incubated at ±28 °C for 48 h. The colonies that emerged after incubation were sub-cultured on NA agar to obtain pure cultures of endophyte bacterial isolates.

4.3. Initial antibacterial screening by dual culture method

A dual culture method was applied for the initial antibacterial activity screening of endophyte bacteria against ATCC strains. Briefly, each bacterial target was cultured in a nutrient broth (NB) medium, shaking at 120 rpm, ±37°C for 24 h. Furthermore, 1.5 % (v/v) of each bacterial culture (about 1×10^5 CFU/mL) was further inoculated into the molten NA plate medium and waited for the media to solidify. Subsequently, fresh bacterial endophytes (24-h incubation) were spot inoculated to that medium followed by incubation at ±37 °C for 24 h. Observations were then made on the inhibition zone formed around the endophytic bacterial colonies.

4.4. Extracellular metabolites extraction

The most potential isolates of bacterial endophytes based on the dual culture method were chosen for metabolic extraction. Briefly, overnight pre-cultured of selected bacterial endophytes 1 % (v/v) in NB medium was inoculated to 1 L of NB medium followed by incubation with shaking at 120 rpm for 3 days at ±28 °C. Furthermore, an equal volume of ethyl acetate 1:1 (v/v) was added to the cultured broth and vigorously shaken for 60 min. The ethyl acetate solvent phase was further separated and evaporated at 50°C to acquire the bacterial extract. The dried extract obtained from the bacterial sample was weighed and stored at 4°C for further use.

4.5. Antibacterial activity by disc diffusion method

The disc diffusion method was conducted for antibacterial tests of selected bacterial extract [40]. Shortly, 1.5% of each bacterial tested suspension (24-h incubation) was inoculated to the Mueller Hinton Agar (MHA) medium, poured into the sterile plate, and allowed to solidify. The extract (10 mg/mL) was dissolved separately in dimethyl sulfoxide (DMSO). Further, 20 µL of the extract was exposed to the sterile filter paper discs (6 mm in diameter), then placed on the surface of the inoculated agar plate. The petri dishes were placed in an oven at ±37 °C for 24 h. Antibacterial tests of the extracts against the test bacterial strains were represented by a growth clear zone of inhibition (mm) near each disc. Tetracycline (100 µg/mL) and 99% DMSO were used for the positive and negative control, respectively.

4.6. Determination of MIC and MBC values

MIC value was performed using a serial micro-dilution method [40]. 100.000 µg/mL stock solution of extract was diluted into concentrations of 15000, 7500, 3750, 1875, 937.5, 468.75, 234.37, and 117.18 µg/mL in 96 sterile well plates. Each extract concentration was mixed with Muller Hinton Broth (MHB) in equal volumes to make a total volume of 200 µL. Subsequently, 100 µL of each bacterial tested (24-h incubation) were adjusted to McFarland standard 0.5 in 0.85% NaCl (equivalent to 1×10^8 CFU/mL) and added to each well to make a final volume of 200 µL followed by incubation at ±37 °C for 24 hours prior to being observed. The MIC of bacterial extracts was determined by observing the turbidity and clarity of each well. MBC value was determined as the lowest concentration of the extract, which totally killed bacterial cells. Tetracycline in range concentration from 500 to 3.9 and 10% DMSO was utilized for the positive and negative control, respectively.

4.7. Total phenolic (TPC) and flavonoid content (TFC)

TPC and TFC were carried out using the Folin-Ciocalteu colorimetric and Aluminum chloride method, respectively [41]. The TPC and TFC were expressed as mg gallic acid equivalent (GAE)/mg extract and mg quercetin equivalent (QE)/mg extract. In short, the TPC reaction was prepared by mixing 0.5 mL of sample (1000 µg/mL), 3.5 mL distilled water, and 0.25 mL of the Folin-Ciocalteu reagent followed by homogenization and incubation at ±28 °C for 8 min. Further, 0.75 mL of 20% sodium bicarbonate was supplemented and incubated again at room temperature for 2 h. Soon after that, the absorbance was further determined using the ELISA reader Thermo Scientific Varioskan Flash (ThermoFischer) at 765 nm. On the other hand, the TFC reaction was prepared by mixing 0.5 mL sample (1000 µg/mL), 2.45 mL of distilled water, 0.15 µL of 5% $NaNO_2$, homogenized and incubated at ±28 °C for 2 min. Subsequently, the mixtures were supplemented with 0.15 mL of 10% aluminum chloride and incubated at ±28 °C for 8 min. Finally, 2 mL of 1M NaOH was added, and the absorbance was measured at 510 nm.

4.8. 1,1-difenil-2-pikrilhidrazil (DPPH) and 2,2'-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) radicals scavenging assay.

DPPH and ABTS radical activity were determined following the previous method [41]. Shortly, sample solutions were diluted in 96 well plates, and 100 µL of 2.5 µM DPPH in methanol was added and incubated at the dark condition for 30 min at ±28 °C. Absorbance was determined at 515 nm. In the ABTS assay, this radical was prepared by mixing 2.45 mM Potassium Persulfate ($K_2S_2O_8$) and 7 mM ABTS solution at 1:1 v/v and incubated in the dark for 12-16 h at ±28 °C. ABTS radical was used until an absorbance value of 0.6 – 0.7 (wavelength of 734 nm). An amount of 150 µL of ABTS radical and 50 µL sample was mixed and incubated in the dark for 30 minutes at ±28 °C, followed by the measurement at 734 nm. The percentage of inhibition of these assays was determined by the formula: [(Abs. control - Abs. sample)/Abs. control] × 100. The inhibition value was then measured in the linear regression equation to acquire the 50% value (IC_{50}). In both DPPH and ABTS assays, Trolox and Ascorbic Acid were used for positive controls, and methanol was applied as a control.

4.9. Cell lines and Cytotoxic activity

In this assay, we used two cell lines model including the human breast adenocarcinoma MCF-7 and human liver carcinoma HepG2 cell lines (ATCC; Rockville, MD, USA). The cell lines were routinely maintained in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C on DMEM High glucose medium completed with 1% antibiotics (penicillin/ streptomycin) and 10% fetal bovine serum. Cytotoxicity assay was carried out by seeding both MCF-7 and HepG2 cells on a 96-well sterile plate at an initial concentration of 1×10^4 cells per well followed by incubation for 24 h. Further, DMEM media were replaced and extract with the final concentration of 100 µg/mL (diluted on DMSO) was added followed by 48 h incubation. Subsequently, 10 µL of (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) stock solution (0.5 mg/ml) was added, followed by 3 h incubation at 37°C. After the complete dissolving of formazan blue with 99% DMSO, cell viability was measured at 570 nm, in which Percent inhibition was determined using the formula: [1- (Abs. sample - Abs. DMSO control)] × 100%. Cisplatin 100 µg/mL (Sigma) and DMSO at a final concentration of 0.05% were utilized for the positive and negative control, respectively.

4.10. Molecular identification and phylogenetic analysis

The genomic DNA of selected endophytic bacteria was isolated using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) as per the manufacturer's instructions. The obtained genomic DNA was analyzed using 1% agarose gel electrophoresis, and the bands were observed. The isolated DNA was used as a template to amplify the 16S rRNA regions through PCR using universal primers for amplifying about 1300 bp: 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′) developed by Marchesi et al. [42]. PCR was performed using GoTaq Green Master Mix (Promega), 4 µl of template DNA (100 ng/ µl), 5 µl of each primer (10 pmol), 25 µl of 2× GoTaq Green Master Mix (Promega) and adjusted with 11 µl of nuclease-free water. PCR amplification was carried out in Applied Biosystem™ 2720. The thermo-cycling conditions involved pre-denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min 45 s, final extension at 72 °C for 10 min. The PCR amplification products were analyzed by gel electrophoresis on 1% agarose (w/v) and the remaining volume was sent for sequencing to the services of FirstBase, Malaysia. The 16S rRNA sequences were compared for identification with the sequence database using BLASTN at https://www.ncbi.nlm.nih.gov. Phylogenetic tree was constructed in the MEGA version 11 application by the neighbor-joining method with 1000x bootstrap replications.

4.11. GC-MS procedure

GC-MS analysis was carried out using an instrument with the specification of Agilent 19091S-433: 93.92873 GC-MS. We utilized an amount of 1 µL sample, which dissolved in n-hexane and injected into the column of HP-5MS 5% Phenyl Methyl Silox 0 °C-325 °C (325 °C): 30 m x 250 µm x 0.25 µm. In detail, helium gas was used as a carrier agent with a 1 mL/min flow rate condition. The initial temperature of 40 °C gradually over 30 minutes to 300 °C. Pressure was set to 7.0699 psi with an average velocity of 36.262 cm/sec and hold time of 1.3789. finally, MSD Chem-Station Data Analysis software incorporated into the instrument was used to analyze the GC-MS results.

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REFERENCES

[1] Megawati, Darmawan A. Resorcinol compounds isolated form the bark of *Myristica fatua* Houtt. Indones J Pharm. 2017;28(2):82-90. https://doi.org/10.14499/indonesianjpharm28iss2pp82.

[2] Lim TK. *Myristica fatua*, In: Edible Medicinal and Non-Medicinal Plants, 2012: 572-574. https://doi.org/10.1007/978-94-007-2534-8_79.

[3] Ashokkumar K, Simal-Gandara J, Murugan M, Dhanya MK, Pandian A. Nutmeg (*Myristica fragrans* Houtt.) essential oil: A review on its composition, biological, and pharmacological activities. Pytotherapy Res. 2022;36(7):2839-2851. https://doi.org/10.1002/ptr.7491.

[4] Muchtaridi, Subarnas A, Apriyantono A, Mustarichie R. Identification of compounds in the essential oil of nutmeg seeds (*Myristica fragrans* Houtt.) that inhibits locomotor activity in mice. Int J Mol Sci. 2010;11(11):4771-4781. https://doi.org/10.3390/ijms11114771.

[5] Fajriah S, Darmawan A, Megawati, Hudiyono S, Kosela S. Hanafi M. New cytotoxic compounds from *Myristica fatua* Houtt leaves against MCF-7 cell lines. Phytocem Lett. 2017;20(92017):36-39.

http://dx.doi.org/10.1016/j.phytol.2017.03.013.

[6] Vivekar MR, Chandrashekar KR. Antioxidant and antibacterial activities of *Myristica fatua* var. Magnifica (Beddome) Sinclair. Asian J Pharm Clin Res. 2016;9(4): 235-239

[7] Prabha B, Sini S, Sherin DR, Neethu S, Rameshkumar KB, Manojkumar TK, Jayamurthy P, Radhakrishnan KV. Promalabaricone B from *Myristica fatua* Houtt. seeds demonstrate antidiabetic potential by modulating glucose uptake via the upregulation of AMPK in L6 myotubes. Nat Prod Res. 2019;35(5):1-6. https://doi.org/10.1080/14786419.2019.1607852

[8] Padmaja V, Srinivas NA, Eswaraiah CM. Evaluation of pharmacological activities of leaves and bark of *Myristica fatua* var. magnifica extracts. Iranian J Pharm Sci. 2019;15(1):39-50. https://doi.org/10.22037/ijps.v15.40596

[9] Strobel G. The emergence of endophytic microbes and their biological promise. J Fungi. 2018;4(2):1-19. https://doi.org/10.3390/jof4020057

[10] Christina A, Christapher V, Bhore SJ. Endophytic bacteria as a source of novel antibiotics: an overview. Pharmacognosy Rev. 2013;7(13):11-16. https://dx.doi.org/10.4103/0973-7847.112833

[11] Tavarideh F, Pourahmad F, Nemati M. Diversity, and antibacterial activity of endophytic bacteria associated with medicinal plant, *Scrophularia striata*. Vet Res Forum. 2022;13(3):409-415. https://doi.org/10.30466%2Fvrf.2021.529714.3174

[12] Urumbil SK, Jesy EJ, Anilkumar M. Antimicrobial and antioxidant potential of endophytic bacteria isolated from *Emilia sonchifolia*. Biosci Biotechnol Res Comm. 2020;13(2):620-626**.** http://dx.doi.org/10.21786/bbrc/13.2/39.

[13] Stelmasiewicz M, Świątek Ł, Ludwiczuk A. Phytochemical profile and anticancer potential of endophytic microorganisms from liverwort species, *Marchantia polymorpha* L. Molecules. 2022;27(1):153. https://doi.org/10.3390/molecules27010153

[14] Munakata Y, Spina R, Slezack-Deschaumes S, Genestier J, Hehn A, Laurain-Mattar D. Screening of endophytic bacteria of *Leucojum aestivum* 'gravety giant' as a potential source of alkaloids and as antagonist to some plant fungal pathogens. Microorganisms. 2022;10(10):2089. https://doi.org/10.3390/microorganisms10102089.

[15] Trung DQ, Hang NTT, Van DM, Ngoc PB, Anh LT. Screening of endophytic bacteria isolated from weed plant to biocontrol stem rot disease on pitaya (*Hylocereus undatus*). Brazilian Archives Biol Technol. 2022;65:1-12. https://doi.org/10.1590/1678-4324-2022200749.

[16] Munif A, Nursalim M, Pradana AP. The potential of endophytic bacteria isolated from *Tagetes* sp.to control *Meloidogyne* spp. infection on tomato plants. Biodiversitas. 2021; 22(96):3229-3236. https://doi.org/10.13057/biodiv/d220626

[17] Afzal I, Shinwari ZK, Sikandar S, Shahzad S. Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. Microbiol Res. 2019;221:36-49. https://doi.org/10.1016/j.micres.2019.02.001.

[18] Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens. 2021;10(2):165. https://doi.org/10.3390/pathogens10020165

[19] Irawan C, Putri ID, Sukiman M, Utami A, Ismail, Putri RK, Lisandi A, Pratama AN. Antioxidant activity of DPPH, CUPRAC, and FRAP methods, as well as activity of alpha-glucosidase inhibiting enzymes from *Tinospora crispa* (L.) stem ultrasonic extract. Pharmacog J. 2022;14(5):511-520. https://doi.org/10.5530/pj.2022.14.128.

[20] Nordin ML, Kadir AA, Zakaria ZA, Abdullah R, Abdullah MNH. *In vitro* investigation of cytotoxic and antioxidative activities of *Ardisia crispa* against breast cancer cell lines, MCF-7 and MDA-MB-231. BMC Compl Alt Med. 2018;18(1):87. https://doi.org/10.1186/s12906-018-2153-5

[21] Gomila M, Mulet M, Garcia-Valdes E, Lalucat J. Genome-based taxonomy of the genus *Stutzerimonas* and proposal of *S. frequens* sp. nov. and *S. degradans sp*. nov. and emended descriptions of *S. perfectomarina* and *S. chloritidismutans*. Microorganisms. 2022;10(7):1363. https://doi.org/10.3390/microorganisms10071363.

[22] Kumar A, Kumar SPJ, Chintagunta AD, Agarwal DK, Pal G, Singh AN, Shimal-Gandara J. Biocontrol potential of *Pseudomonas stutzeri* endophyte from *Withania somnifera* (Ashwagandha) seed extract against pathogenic *Fusarium oxysporum* and *Rhizoctonia solan*i. Arch Phytopathol Plant Protect. 2022;55(1):1-18. https://doi.org/10.1080/03235408.2021.1983384.

[23] Szymanska S, Lis MI, Piernik A, Hrynkiewicz K. *Pseudomonas stutzeri* and *Kushneria marisflavi* alleviate salinity stress-asssociated damages in barley, lettuce, and sunflower. Front Microbiol. 2022;13:788893. https://doi.org/10.3389/fmicb.2022.788893

[24] Dhanya KI, Swati VI, Venka KS, Osborne WJ. Antimicrobial activity of *Ulva reticulata* and its endophytes. J Ocean Univ China. 2016;15:363-369. http://dx.doi.org/10.1007/s11802-016-2803-7

[25] Rojas-Solis D, Zetter-Salmon E, Contreras-Perez M, Rocha-Granados MdC, Macias-Rodriguez L, Santoyo G. *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 endophytes produce antifungal volatile organic compounds and exhibit additive plant growth-promoting effects. Biocatal Agric Biotechnol. 2018;13: 46-52. https://doi.org/10.1016/j.bcab.2017.11.007.

[26] Uzair B, Ahmed N, Ahmad VU, Mohammad FV, Edward DH. The isolation, puri¢cation and biological activity of a novel antibacterial compound produced by *Pseudomonas stutzeri*. FEMS Microbiol Lett. 2008;279:243-250. https://doi.org/10.1111/j.1574-6968.2007.01036.x

[27] Tungmunnithum D, Thongboonyou A, Pholboon A, Yangsabai A. Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: An overview. Medicines (Basel). 2018;5(3):93. https://doi.org/10.3390%2Fmedicines5030093
[28] Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: An overview. The Sci World J. 2013; 2013: 1-16. http://dx.doi.org/10.1155/2013/162750.
[29] Varsha KK, Devendra L, Shilpa G, Priya S, Pandey A, Pandey A, Nampoothiri KM. 2,4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp. Int J Food Microbiol. 2015; 211:44-50. https://doi.org/10.1016/j.ijfoodmicro.2015.06.025.
[30] Chawawisit K, Bhoopong P, Phupong W, Lertcanawanichakul M. 2, 4-Di-tert-butylphenol, the bioactive compound produced by *Streptomyces* sp. KB1. J Appl Pharm Sci. 2015;5(3):7-12. https://dx.doi.org/10.7324/JAPS.2015.510.S2
[31] Mishra R, Kushveer JS, Khan MIK, Pagal S, Meena CK, Murali A, Dhayalan A, Sarma VV. 2,4-di-tert-butylphenol isolated from an endophytic fungus, *Daldinia eschscholtzii*, reduces virulence and quorum sensing in *Pseudomonas aeruginosa*. Front Microbiol. 2020;11:1668 https://doi.org/10.3389/fmicb.2020.01668.
[32] Kiran GS, Priyadharsini S, Sajayan A, Ravindran A, Selvin J. An antibiotic agent pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro isolated from a marine bacteria *Bacillus tequilensis* MSI45 effectively controls multi-drug resistant *Staphylococcus aureus*. RSC Advances. 2018;8:17837–17846. https://doi.org/10.1039/C8RA00820E
[33] Ser HL, Palanisamy UD, Yin WF, Malek SNA, Chan KG, Goh BH, Lee LH. Presence of antioxidative agent, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- in newly isolated *Streptomyces mangrovisoli* sp. nov. Front Microbiol. 2015; 6:854. https://doi.org/10.3389/fmicb.2015.00854
[34] Rajivgandhi G, Vijayan R, Maruthupandy M, Vaseeharan B, Manoharan N. Antibiofilm effect of *Nocardiopsis* sp. GRG 1 (KT235640) compound against biofilm forming Gram negative bacteria on UTIs. Microb Pathog. 2018;118:190-198. https://doi.org/10.1016/j.micpath.2018.03.011.
[35] Prastya ME, Astuti RI, Batubara I, Takagi H, Wahyudi AT. Chemical screening identifes an extract from marine *Pseudomonas* sp.-PTR-08 as an anti-aging agent that promotes fssion yeast longevity by modulating the Pap1–ctt1+ pathway and the cell cycle. Mol Biol Rep. 2020;47:33-43. https://doi.org/10.1007/s11033-019-05102-0.
[36] Javed MR, Salman M, Tariq A, Tawab A, Zahoor MK, Naheed S, Shahid M, Ijaz A, Ali H. The antibacterial and larvicidal potential of bis-(2-ethylhexyl) phthalate from *Lactiplantibacillus plantarum*. Molecules. 2022;27(21):1-15. https://doi.org/10.3390/molecules27217220.
[37] Habib MR, Karim MR. Antimicrobial, and cytotoxic activity of di-(2-ethylhexyl) phthalate and anhydrosophoradiol-3-acetate isolated from *Calotropis gigantea* (Linn.) Flower. Mycobiology. 2009;37(1):7220. https://doi.org/10.4489/myco.2009.37.1.031
[38] Cruz-Ramirez SG, Lopez-Saiz CM, Rosas-Burgos EC, Cinco-Moroyoqui FJ, Velazquez C, Hernandez J, Burgos-Hernandez A. Antimutagenic bis (2-ethylhexyl) phthalate isolated from octopus (*Paraoctopus vulgaris*). Food Sci Technol. 2021;41(2):314-320. https://doi.org/10.1590/fst.26119.
[39] Singh R, Pandey KD, Singh M, Singh SK, Hasem A, Al-Arjani ABF, Abd_Allah EF, Singh PK, Kumar A. Isolation, and characterization of endophytes bacterial strains of *Momordica charantia* L. and their possible approach in stress management. Microorganisms. 2021;10(2):290. https://doi.org/10.3390/microorganisms10020290.
[40] Priyanto JA, Prastya ME, Sinarawadi GS, Datu'salamah W, Avelina TY, Yanuar AIA, Azizah E, Tachrim ZP, Mozef T. The antibacterial and antibiofilm potential of *Paederia foetida* Linn. leaves extract. J Appl Pharm Sci. 2022;12(10):117-124. http://dx.doi.org/10.7324/JAPS.2022.121012.
[41] Prastya ME, Astuti RI, Batubara I, Wahyudi AT. Antioxidant, antiglycation and in vivo antiaging efects of metabolite extracts from marine sponge-associated bacteria. Ind J Pharm Sci. 2019;81:344-353. http://dx.doi.org/10.36468/pharmaceutical-sciences.516
[42] Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, Wade WG. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol. 1998;64(2):795-799. https://doi.org/10.1128/aem.64.2.795-799.1998