Endophytic fungus *Fusarium* sp. MCXR isolated from the fruit pulp of *Annona muricata* L. and its cytotoxic effect on MCF-7 breast cancer cells

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Abstract:

Annona muricata* (*A. muricata*, Annonaceae) is a medicinal plant containing diverse bioactive compounds, including acetogenins, which have been widely investigated for their cytotoxic and anticancer potential. Endophytic fungi associated with *A. muricata* may represent an additional source of bioactive metabolites; however, information on endophytic fungi residing in soursop fruit remains limited. This study aimed to isolate and identify endophytic fungi from the fruit pulp of *A. muricata* grown in Vietnam, and preliminarily evaluate the cytotoxic activity of the fungal crude extract. A fungal isolate, designated MCXR, was obtained and identified as *Fusarium* sp. based on macroscopic and microscopic morphological characteristics together with internal transcribed spacer (ITS) DNA sequence analysis. The fungal ethanolic crude extract showed a positive qualitative reaction with Kedde's reagent, suggesting the possible presence of acetogenin-related compounds. Its cytotoxic activity was evaluated *in vitro* against the human breast cancer cell line MCF-7. The extract inhibited MCF-7 cell proliferation in a concentration-dependent manner, with an IC_{50} value of 134 µg/ml. These findings suggest that *A.

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***muricata*-associated endophytic fungi may represent a potential source of bioactive metabolites. Further studies are required to chemically identify the active compounds, assess their selectivity, and elucidate their mechanisms of action.**

Keywords: **acetogenins, *Annona muricata,* anticancer activity, breast cancer, endophytic fungi.**

***Classification numbers*: 3.3, 3.4**

1. Introduction

Cancer treatment remains complex and challenging. Common treatment modalities include chemotherapy, radiotherapy, tumour surgery, immunotherapy, and hormonal therapy. However, these approaches are often associated with severe side effects and are not always fully effective [1-4]. This highlights the need for improved cancer therapies and new, effective and affordable anticancer compounds with fewer side effects. Breast cancer is the most prevalent cancer among women worldwide. According to the World Health Organization, approximately 2.3 million women were diagnosed with breast cancer globally in 2020, resulting in 685,000 deaths. Breast cancer remains a major health concern for women, particularly in Asian countries [5]. In Vietnam, breast cancer ranks among the most common cancers and is a leading cause of death. The Global Cancer Organization reported that Vietnam recorded more than 21,000 new breast cancer cases and 9,000 related deaths in 2020.

Natural products are rich sources of therapeutic agents. *Annona muricata* L. (*A. muricata* L.), commonly known as soursop (also referred to as graviola or guanabana), is a small evergreen tropical tree reported to occur in Central and South America as well as Vietnam. Extracts from *A. muricata* L. contain compounds with potential therapeutic properties [6, 7]. The use of *A. muricata* in cancer treatment has been reported in several regions worldwide. A study conducted in Jamaica on cancer patients' views regarding the use of medicinal plants for self-medication found that 80% of participants reported using *A. muricata* to treat various malignancies, particularly breast and prostate cancer [7]. In addition, 80.9% of patients with breast, prostate, or colorectal cancer attending specialty oncology clinics in Trinidad were reported to use *A. muricata* [8]. A cross-sectional study suggests that 14% of Peruvian patients with liver cancer have used *A. muricata* to treat their cancer-related symptoms [9]. An investigation of an ionic liquid extract of

soursop against human breast cancer cells (MCF-7) showed that the extract inhibited tumour cell growth [10].

Acetogenins are among the major bioactive compounds found in different parts of *A. muricata* L., including the roots, stem bark, leaves, pulp, peel, and seeds. Compounds such as annonacin and bullatacin have been isolated using various methods. Acetogenins are classified as polyketides, a large family of natural products biosynthesised via the polyketide pathway. Structurally, they are unique secondary metabolites characterised by a C35/C37 carbon skeleton derived from the combination of a long-chain C32/C34 fatty acid with a 2-propanol unit, forming an α, β-unsaturated γ-lactone ring with a methyl substituent at the C-2 position. In addition to the lactone moiety, these compounds may contain one or more tetrahydrofuran (THF) or tetrahydropyran (THP) rings, as well as epoxy and hydroxyl groups along the long aliphatic chain, which contribute to their structural diversity and biological activities [11]. Acetogenin yields are often low and highly dependent on extraction conditions [12]. Acetogenins have demonstrated several biological activities, including antioxidant, antimicrobial, and cytotoxic effects against certain cancer cell lines [13]. S.U.F.S. Najmuddin, et al. (2016) [14] investigated *A. muricata* samples from different locations for activity against breast cancer cell lines, including MCF-7, MDA-MB-231, and 4 T1. The aqueous extract of *A. muricata* leaves collected from Selangor, Malaysia, showed the most potent effect, with IC_{50} values of 220, 350, and 250 µg/ml against these breast cancer cell lines, respectively. An *in vivo* study in female albino Sprague-Dawley rats reported a reduction in the proliferative index of DMBA-induced breast cancer, with the most significant effect at 300 mg/kg [15].

Plant endophytic microbes commonly establish symbiotic interactions with their hosts and can produce bioactive compounds with potential for drug development [16]. Several plant endophytic fungi have been reported to produce anticancer compounds, and their bioactive metabolites have exhibited cytotoxic activity against various cancer cell lines [17-19]. A systematic review by I.M.M. Silva, et al. (2024) [20] summarised studies reporting endophytic fungi in soursop leaves and showed that extracts from some of these fungi suppressed cancer cell proliferation while exhibiting low toxicity to normal cells [21-24]. However, information on endophytic fungi in soursop fruit, their potential to produce bioactive compounds, and the

cytotoxic activity of their extracts remains limited. Therefore, the present study aimed to isolate endophytic fungi from the pulp of soursop fruit grown in Vietnam, characterise the fungal isolate based on macroscopic and microscopic morphology and ITS DNA sequence analysis, and preliminarily screen the fungal crude extract for cytotoxic activity against breast cancer cells.

2. Materials and methods

2.1. Materials

Soursop fruits were harvested from disease-free plants in Da Lat City, Vietnam, placed in clean plastic bags, and protected from heat and light until use. MCF-7 human breast cancer cells were kindly provided by Prof. J. M. Pezzuto (Long Island University, USA) and Prof. Jeanette Maier (University of Milan, Italy). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine, sodium pyruvate, $NaHCO_3$, penicillin/streptomycin, 5% fetal bovine serum (FBS), and 0.05% trypsin-EDTA.

2.2. Isolation of endophytic fungi

Endophytic fungi were isolated from the fruit pulp of *A. muricata* following J. Hallmann, et al. (2006) [25], with modifications. The fruits were thoroughly washed with clean water to remove adhering surface debris and microorganisms and then rinsed three times with sterile distilled water. Surface sterilisation was performed by immersing the fruits in 5% sodium hypochlorite solution for 5 min in a sterile container, followed by three rinses with sterile distilled water and immersion in 70% ethanol for 5 min. The surface-sterilised fruits were placed on sterile paper and allowed to dry completely.

After removal of the peel, the fruit pulp was cut into small pieces and placed on potato dextrose agar (PDA; TM Media, India) supplemented with streptomycin (100 µg/ml) to suppress bacterial growth. The plates were incubated at 28°C for 10 days and monitored for fungal growth emerging from the internal tissues. Fungal isolates were purified by repeated subculturing on PDA and stored using silica gel at -20°C for subsequent studies.

2.3. Preparation of fungal crude extract and qualitative screening

The fungal crude extract was prepared according to A. Grzybowski, et al. (2012) [26], with modifications. The fungal isolate was cultured on PDA at 28°C for 7 days. To recover the secreted exudate, the entire agar content was transferred to a 50 ml Falcon tube containing 20 ml

of 0.9% NaCl and stored at -20°C overnight. The following day, the liquid fraction was decanted and filtered through filter paper. The filtrate was partially purified using ethanol at concentrations of 20% and 50%. After centrifugation, the resulting liquid fraction was dried at 60°C to obtain the extract residue. The residue was divided into two portions and dissolved separately in 0.9% NaCl and DMSO. Both preparations were stored at -20°C until use and were designated as fungal ethanolic crude extracts.

Acetogenins were qualitatively screened using the method described by G. Aguilar-Hernández, et al. (2020) [27]. Briefly, 1 ml of fungal crude extract prepared in 0.9% NaCl was mixed thoroughly with 0.5 ml of solution A (2% 3,5-dinitrobenzoic acid in 90% ethanol) and 0.5 ml of solution B (5% NaOH in deionised water), collectively referred to as Kedde's reagent. The development of a cherry-red colour was considered a positive reaction.

2.4. Cytotoxic activity test

The cytotoxic activity of the fungal crude extract against MCF-7 human breast cancer cells was evaluated using the sulforhodamine B (SRB) assay according to P. Skehan, et al. (1990) [28]. MCF-7 cells were maintained in the culture medium described in Section 2.1. Approximately 2×10^4 cells were seeded per well in 96-well plates and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 18-20 h before treatment.

The cells were then treated with the fungal crude extract at five concentrations (1.6, 8, 40, 200, and 1000 µg/ml) and incubated for an additional 48 h. For the day-0 control, the test extract was replaced with 1% DMSO, and the cells were fixed after 1 h with 20% trichloroacetic acid. After 48 h of treatment, cells in the experimental wells were also fixed with 20% trichloroacetic acid and stained with sulforhodamine B at a final concentration of 0.4% for 30 min. The dye was removed, and the wells were washed three times with 1% acetic acid. Subsequently, 200 µl of 10 mM Tris base was added to each well, and absorbance at 540 nm (OD_{540}) was measured using an ELISA plate reader (BioTek). All experiments were performed in triplicate. Ellipticine at 0.08, 0.4, 2, and 10 µg/ml was used as the reference control, whereas DMSO was used as the negative control at a final concentration of 0.05% in the test wells.

The SRB assay estimates total cellular protein based on the amount of protein-bound dye, with absorbance being proportional to cell biomass. The percentage of cell growth inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 100 - \frac{\text{OD(sample)} - \text{OD(day0)}}{\text{OD(DMSO)} - \text{OD(day0)}} x100$$

where OD(sample) is the mean OD of wells treated with the fungal crude extract, OD(DMSO) is the mean OD of the DMSO negative-control wells, and OD(day0) is the mean OD of the DMSO control wells fixed after 1 h. The IC_{50} value, defined as the concentration required to inhibit cell growth by 50%, was determined using TableCurve 2D v4 software.

2.5. Colony morphology and microscopic characterisation of the endophytic fungus

The endophytic fungal isolate obtained from *A. muricata* fruit pulp was characterised on the basis of macroscopic and microscopic morphological features. Colony characteristics, including colour, texture, margin, and diameter, were recorded. Microscopic features, including spores, conidiophores, and hyphae (mycelium), were examined using the 40× and 100× objectives. The fungal isolate was cultured on PDA at 30°C with an inclined coverslip for 9 days. After incubation, the coverslip was examined using a Nikon SMZ 1270 microscope (Nikon, Japan). Morphological characterisation was subsequently complemented by molecular identification and sequencing.

2.6. Molecular identification

Molecular identification of the endophytic fungal isolate was performed by sequencing the ITS region. Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The ITS region was amplified using the universal primers ITS1 (forward, 5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (reverse, 5′-TCC TCC GCT TAT TGA TAT GC-3′) (MWG-Biotech AG, Germany) in a T100™ Thermal Cycler (Bio-Rad). PCR was performed in a total volume of 25 µl containing 12.5 µl of 2× MyTaq Mix (Bioline), 1 µl of each primer (10 pmol/µl), 1 µl of genomic DNA (50 ng/µl), and 9.5 µl of ddH_2O.

PCR amplification consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for

1 min, with a final extension at 72°C for 7 min. The PCR product was analysed by electrophoresis on a 1% agarose gel and sequenced by First BASE Laboratories (Malaysia). The resulting ITS sequence was compared with sequences available in the NCBI GenBank database using BLASTN and further compared with the UNITE database. A phylogenetic tree was constructed using the neighbour-joining method based on the Kimura two-parameter model with 1,000 bootstrap replicates in MEGA v7.0 [29]. The obtained sequence was deposited in GenBank, and an accession number was assigned.

3. Results and discussion

3.1. Endophytic fungal isolation

Previous studies on total acetogenin extraction from different parts of *A. muricata* L. (soursop) fruit showed that the seeds contain the highest concentration of acetogenins, followed by the pulp and peel [27]. Endophytic fungi isolated from different parts of *A. muricata* have been reported to include genera such as *Aspergillus*, *Penicillium*, and *Neocosmospora* [20]. In this study, an endophytic fungus was isolated from the pulp (Fig. 1B) of *A. muricata* fruit grown in Vietnam. The endophytic fungal isolate was purified by repeated streaking on PDA medium and designated MCXR.

The MCXR isolate grew steadily on PDA medium at a relatively slow growth rate, reaching a colony diameter of 40 to 45 mm after 11-14 days (Fig. 1C). Colony morphology changed markedly over time. The colony was cottony, dense, and cream-coloured, with an opaque white, ring-shaped margin, and no pigment was observed in the agar. The hyphae were curly and cream-coloured (Fig. 1C). The spores were observed after 9 days of cultivation. Microscopically, the spores occurred singly or in clusters along the hyphae and at branch tips. Microconidia predominated and were elliptical or rod-shaped (slightly cylindrical), with smooth surfaces. Some contained one to two thin-walled septa (Fig. 1D). Based on the morphological characteristics observed in this study and comparison with the literature, MCXR was tentatively assigned to the genus *Fusarium*. The subsequent evaluation was focused on molecular analysis.

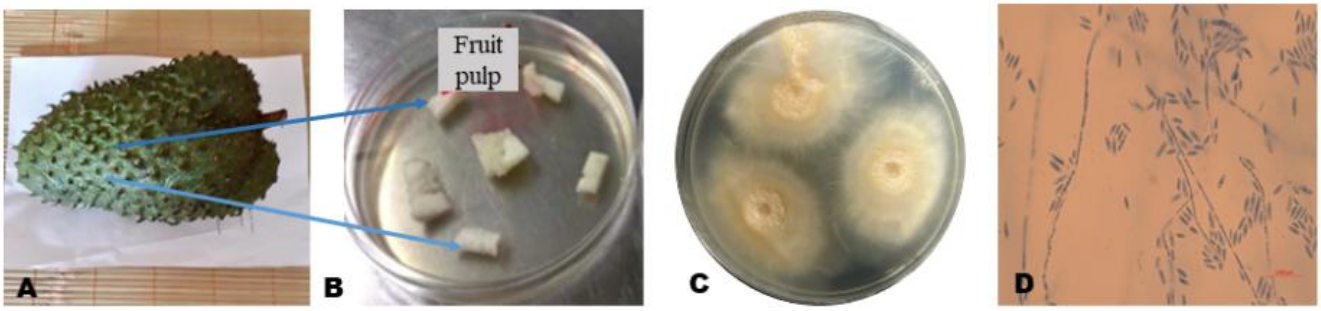


Fig. 1. Isolation of the endophytic fungus MCXR from the pulp (B) of soursop fruit (*Annona muricata* L.) (A); morphology of MCXR (with silica gel on the surface) on PDA medium after 11 days (C), and MCXR fungal spores (D, ×100). Green arrows indicate the pulp inside the soursop fruit.

3.2. Endophytic fungal crude extract and acetogenin-related compounds

The endophytic fungus MCXR was investigated as a potential source of crude extracts containing anticancer compounds. To minimise potential damage to bioactive molecules, the extraction procedure used a physiological buffer (0.9% NaCl), controlled temperature, and ethanol. Fungal exudates secreted onto the culture agar were collected using the simple extraction procedure.

The MCXR crude extract was qualitatively screened with Kedde's reagent, as described by G. Aguilar-Hernández, et al. (2020) [27], to assess the presence of acetogenin-related compounds. Development of a cherry-red colour indicated a positive reaction (Fig. 2, tube A), suggesting the possible presence of acetogenin-related compounds in the crude extract. The compounds were recovered from the fungal culture agar using a simple procedure involving water, controlled temperature, and ethanol (Fig. 2, tube C). These preliminary findings support further chemical characterisation of the fungal extract.

Previous reports showed that acetogenin-related compounds producing strong rose-wine colouration were also detected using thin-layer chromatography plates treated with Kedde's reagent [26, 30]. Although several studies have used solvents such as ethyl acetate and n-hexane to extract bioactive compounds from fungal broth cultures [22, 23, 31], simple procedures using less toxic solvents have also attracted interest. Y.E. Hadisaputri, et al. (2021) [32] reported that a water-derived extract fraction of *A. muricata* leaves exhibited anticancer activity against the MCF-7 breast cancer cell line. A. Grzybowski, et al. (2012) [26] indicated that the acetogenin-

containing crude extract from *A. muricata* seeds using absolute ethanol exhibited lethal effects against *Aedes aegypti* larvae and *Artemia salina* nauplii, with LC_{50} values of 93.48 µg/ml and 10.2 µg/ml, respectively.



Fig. 2. Qualitative spot test of the fungal crude extract using Kedde's reagent for preliminary detection of acetogenin-related compounds. **(A)** Fungal crude extract after reaction with Kedde's reagent (positive cherry-red colour change); **(B)** Negative control (no colour change); **(C)** Fungal crude extract without reagent.

3.3. Cytotoxic activity of MCXR crude extract against MCF-7 breast cancer cells

The cytotoxic activity of soursop leaves has been associated with bioactive compounds such as acetogenins, bullatacin, and squamocin [33]. Among these compounds, acetogenins extracted from *A. muricata* leaves have been widely investigated for their anticancer activity. They have been reported to exert antiproliferative effects on several cancer cell lines and to show no toxicity or harm to normal PDL cells under the examined conditions [13, 33-35]. Endophytic fungi isolated from *A. muricata* have also been reported to exhibit antibacterial activity and inhibit cancer cells [20]. In this study, the MCXR crude extract was tested against MCF-7 human breast cancer cells at different concentrations as a preliminary assessment of its anticancer activity.

The results showed a concentration-dependent increase in cell inhibition. At the lowest tested concentration of 1.6 µg/ml, MCF-7 cells showed 2.9% inhibition. At 200 µg/ml, cell growth was strongly suppressed, with 78.7±1.46% inhibition. At the highest concentration tested, 1000 µg/ml, cell inhibition reached 99.92±2.48%. The crude extract showed an IC_{50} value of 134±7 µg/ml after 48 h of incubation at 37°C (Fig. 3B). In comparison, the positive control, ellipticine, a cell-permeable antitumour alkaloid, exhibited strong inhibition with an IC_{50} value

of 0.36 µg/ml (Fig. 3A). Microscopic observation also showed a clear reduction in viable cell density after treatment with 200 µg/ml crude extract (Fig. 3D) compared with untreated cells (Fig. 3C).

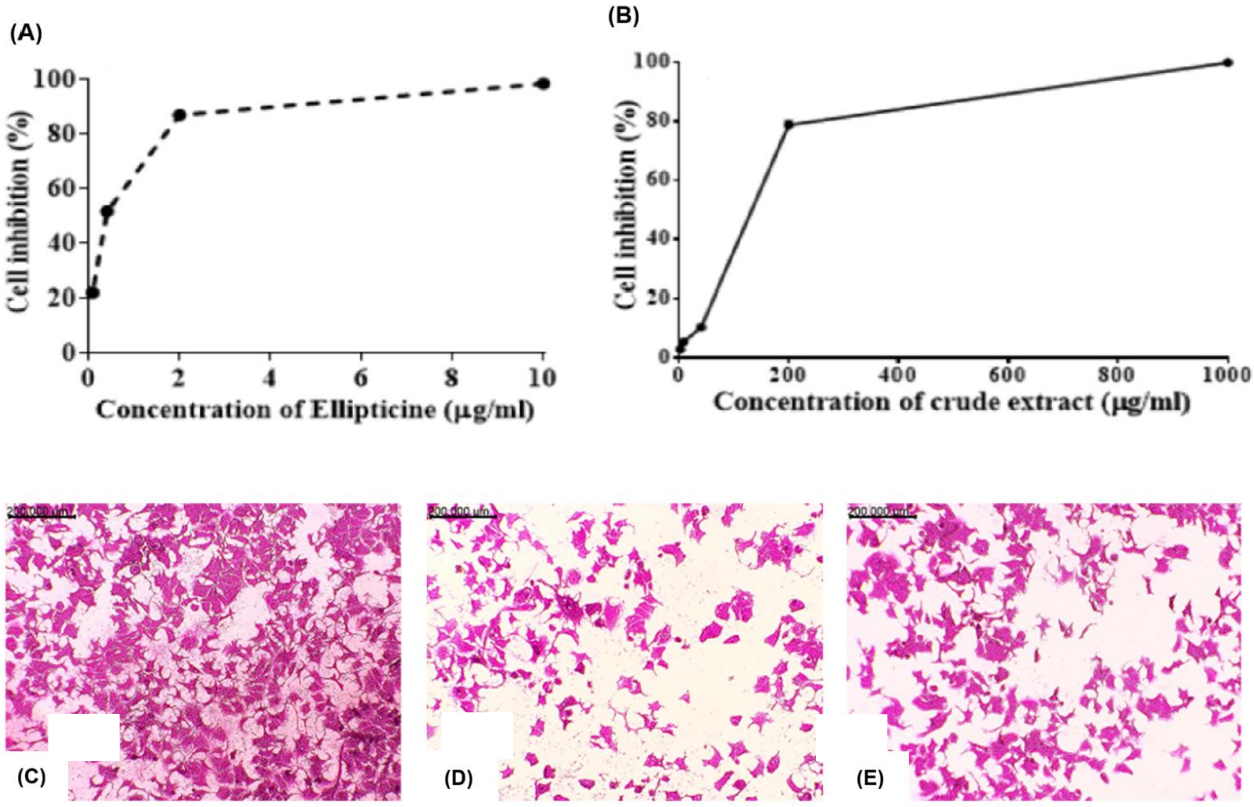


Fig. 3. Cytotoxic effect of the crude extract of *Fusarium* sp. MCXR on the MCF-7 breast cancer cell line. (A) Cell inhibition by the positive control, ellipticine; **(B)** The fungal crude extract against MCF-7 cells; **(C)** Untreated MCF-7 cells; **(D)** MCF-7 cells after 48 h of treatment with 200 µg/ml crude extract, and **(E)** 2 µg/ml ellipticine.

According to the criteria established by A. Subarnas, et al. (2012) [36], IC_{50} values are classified as strong (<100 µg/ml), moderate (101-200 µg/ml), or weak (>201 µg/ml). Accordingly, the IC_{50} value of the MCXR fungal crude extract indicates moderate cytotoxic activity. Minarni, et al. (2017) [22] reported that an ethyl acetate extract of endophytic fungi isolated from *A. muricata* leaves inhibited MCF-7 breast cancer cell growth, with an IC_{50} value of 19.20±7.71 µg/ml. Similarly, F.R. Arifni, et al. (2017) [23] reported an IC_{50} value of 20.80 µg/ml against the WiDr colon cancer cell line for an extract from soursop leaf endophytic fungi.

Thus, the present findings indicate that the MCXR fungal crude extract moderately suppresses MCF-7 cell proliferation.

Previous studies of *A. muricata* leaf extracts have associated reduced cancer-cell viability and proliferation with apoptosis-related mechanisms, including decreased Bcl2 regulation, increased Bax, *caspase-9* and *caspase-3* mRNA expression, inhibition of mitochondrial complex I, and inhibition of ubiquinone-oxidase NADH in the cancer cell plasma membrane [32, 37]. Extracts from endophytic fungi isolated from soursop leaves have also been reported to cause morphological changes, including smaller or shrunken cells and reduced cell density, while showing low toxicity to normal cells [23]. However, these mechanisms were not directly examined in the present study. Further studies should evaluate its selectivity using non-cancerous cell lines, purify and identify the active compounds, and investigate the underlying mechanisms of action. Overall, the MCXR crude extract exhibited moderate, concentration-dependent cytotoxicity against MCF-7 cells.

3.4. Identification of the endophytic fungal isolate

The fungal isolate MCXR was identified by ITS DNA sequence analysis. The ITS DNA amplicon of 830 bp was amplified and sequenced. The obtained sequence comprised the ITS region together with the complete 5.8S rDNA region and partial sequences of the 18S and 28S rDNA. Comparison of the MCXR ITS DNA sequence with sequences available in GenBank showed 100% sequence identity with multiple strains assigned to the genus *Fusarium*. Phylogenetic analysis placed MCXR close to *Fusarium* species, including *F. napiforme* CBS 748.97, *F. napiforme* 2010, *F. napiforme* 2008, and *F. subglutinans* NRRL 66333, with a bootstrap value of 69% (Fig. 4).

To further assess the taxonomic assignment, the ITS sequence was analysed using the UNITE database, which is designed for sequence-based fungal identification and uses the Species Hypothesis (SH) system. The query showed high similarity (99.88-100%) to *Fusarium* sequences. This result was consistent with the GenBank BLAST analysis and supported assignment of the isolate to the genus *Fusarium*. Based on its morphological and microscopic characteristics together with ITS DNA sequence analysis, the isolate was designated *Fusarium*

sp. MCXR. The ITS DNA sequence of *Fusarium* sp. MCXR was deposited into the GenBank database under the accession number PQ285130.

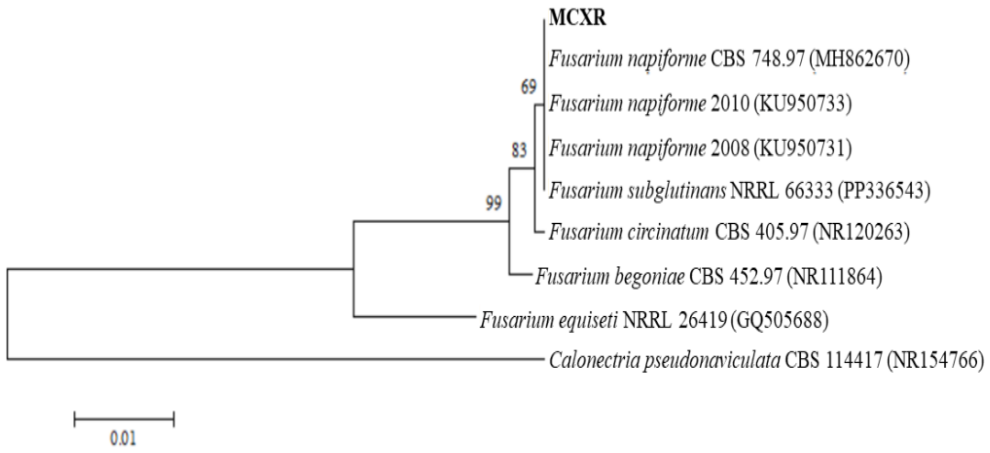


Fig. 4. Phylogenetic position of the endophytic fungus *Fusarium* sp. MCXR based on ITS DNA sequences from MCXR and type strains of closely-related *Fusarium* species. The percentages of taxa clustered together in the bootstrap test (1000 replicates) are shown at the internal nodes.

Several studies have reported that endophytic fungi isolated from different parts of *A. muricata* predominantly belong to the genera *Aspergillus*, *Penicillium*, *Mucor*, *Phomopsis*, and *Rhizoctonia*, with *Aspergillus* being the most frequently observed genus [20]. Evidence regarding anticancer endophytic fungi residing in soursop fruit pulp remains limited. *Fusarium* has not been previously reported in *A. muricata* in the literature considered in this study. However, a systematic review by A.M. Ahmed, et al. (2023) [38] reported that *Fusarium* species are commonly associated with valuable plants and represent a rich source of natural products. In the present study, MCXR was identified as *Fusarium* sp., and its crude extract showed a positive Kedde's reaction and *in vitro* cytotoxic activity against MCF-7 cells. Further studies are required to purify and chemically identify the compounds responsible for this activity and to evaluate their potential applications.

4. Conclusions

This study describes the endophytic fungus *Fusarium* sp. MCXR isolated from the pulp of soursop fruit in Da Lat, Vietnam. The fungal ethanolic crude extract showed a positive qualitative reaction with Kedde's reagent and moderate cytotoxicity against human breast cancer

cells (MCF-7). These findings suggest that endophytic fungi associated with *A. muricata* fruit may represent a potential source of bioactive metabolites. Further chemical characterisation, selectivity assessment, and mechanistic studies are required to identify the active compounds and clarify their anticancer potential.

CRediT author statement

Nguyen Thi Hong Hanh and Nguyen Thi Thanh Loi contributed to the study conception and design, and prepared the manuscript. Nguyen Thi Hong Hanh supervised all experiments conducted by Nguyen Thi Thanh Loi, Duong Thu Huong, Ngo Cao Cuong, and Nguyen Viet Cuong. All authors reviewed the results and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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