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Allium cepa bulbs and *Allium sativum* cloves host different strains of *Fusarium oxysporum* with distinct bioactivities

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Abstract

Plant-fungi interaction serves various insights for research. Therefore, the study aimed to isolate endophytic fungi from *Allium cepa* (onion) bulbs and *Allium sativum* (garlic) cloves to evaluate the biological activity of their metabolites. Remarkably, a heteromorphic strain of *Fusarium oxysporum* was successfully isolated and identified via Internal Transcribed Spacer (ITS) amplicon sequencing: the strain from onion was designated ACE (OR062575), and the one from garlic as ASE (OR062574). Morphologically, ACE spores exhibited no defined shape, whereas ASE produced oval and amygdaloid chlamydospores. The fungal extracts were evaluated using the free radical scavenging DPPH assay, antimicrobial disc diffusion method, and brine shrimp lethality bioassay. Notably, ASE demonstrated approximately 1.5 times higher 50% free radical inhibition compared to ACE. The ACE extract (80 µg/disc) showed significant antibacterial activity against *Escherichia coli* (18 mm) and *Bacillus megaterium* (16 mm), whereas ASE exhibited roughly half this activity at the same dose against *E. coli* and *Pseudomonas aeruginosa*. Interestingly, the cytotoxicity of ACE had a logarithmic 50% lethal dose of 0.44 µm/ml, and no significant difference was observed from the reference vincristine sulfate. These findings highlight the need for further investigation into how different *Allium* species influence fungal genetic variation and metabolic activities.

Introduction

A diverse collection of fungi that live asymptomatically in healthy plant tissues or intercellular spaces is called endophytic fungi (Banerjee, 2011). They support host plant development by directly producing secondary metabolites, which assist plant survival under biotic and abiotic stressors. Many of these metabolites were previously considered to be generated solely by the host (Wen, J. 2022); notably, some of them are therapeutically significant.

Onion (*Allium cepa*) and Garlic (*Allium sativum*) are two of the most effective medicinal spices belonging to the genus *Allium* and the family *Alliaceae* (Mirabeau & Samson, 2012). Several studies have demonstrated that onions, when used as food and spice, help prevent platelet aggregation, enhance immunity, alleviate symptoms of diabetes mellitus and asthma, and lower blood levels of triglycerides, cholesterol, and thromboxanes (Chisty et al., 1996; Gazzani et al., 1998; Sanderson et al., 1999; Shimura et al., 1999). Furthermore, onions have been associated with a reduced risk of certain cancers, including ovarian, breast, gastric, colon, and brain cancers (Hsing et al., 2002; Nicastro et al., 2015; Zhu et al., 2014). Similar to its counterpart, garlic is also beneficial for health. It contains high vitamin C content and possesses important immune-boosting and anticancer properties (Fleischauer et al., 2000; Kyo et al., 2001). It has several bioactivities, including antidiabetic, antihypertensive, antioxidant, anti-atherosclerotic, renoprotective, antibacterial, and antifungal properties. Both onions and garlic are rich in organosulfur compounds—including alliin, allicin, ajoenes, and vinyldithiins—which are believed to be primarily responsible for their biological activity (Benkeblia, 2004; El-Saber Batiha et

al., 2020). Therefore, the spices are a good source to isolate endophytic fungi with potential physical activity.

Investigators isolated *Clonostachys rosea, Trichoderma lixii, Trichoderma asperellum, T. atroviride, T. harzianum, and Fusarium spp*. from onion roots, stems, and leaves (Caruso et al., 2020). A recent study isolated 11 fungal strains from *A. cepa*, and one isolate *Penicillium sp*. exhibited potential anti-infective activity against *Fusarium solani, Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*. The anti-infective activity of onion is believed to be primarily attributed to the presence of polyketides, macrolides, phenolics, and terpenoids (Hassan et al., 2023; Wang et al., 2023). Moreover, the colonization of endophytic fungi protects the onion from pests (Muvea et al., 2014). Despite advances in research on onion-associated endophytes, similar studies focusing on garlic remain limited. However, the fungal and bacterial associations with garlic plant physiology are well established by comprehensive research (Costa Júnior et al., 2020; Mondani et al., 2021). The previous research mainly focused on the two spice plants to relate the pathophysiological connection of the endophytes. This study aimed to investigate the genetic relationships and biological potential of endophytic fungi isolated from fresh tissues of onion bulbs and garlic cloves. Accordingly, we isolated fungi from the fresh tissue of onion bulbs and garlic cloves.

Materials and Methods

Collection of samples

The matured onion and Garlic were collected from a farmland located in Noakhali, Bangladesh (22.70ºN 91.10ºE) in February, 2021. The samples were placed in sterile packets and transported to the laboratory for processing within a few hours.

Isolation of endophytic fungi

Each time, three bulbs of onion and three bulbs of garlic were randomly selected for fungal isolation. The samples were primarily cleaned with water and peeled. Then, the series of surface sterilizations were followed: washing with ethanol (70%) for 1 min, sodium hypochlorite (1.3 M) for 3 min, and ethanol (70%) for 30s. After that, the flesh on the onion bulb and garlic cloves was sliced into smaller pieces in the laminar hood to cultivate fungi on an antibiotic (0.1 mg/ml ciprofloxacin) enriched solid Potato Dextrose Agar (PDA) media. One portion of the sliced samples was left unsterilized and used as control. After 21 days of cultivation, the fully grown fungal mycelium was traced and transferred to a test tube slant following the method described by Baral et al (2024) (Baral et al., 2024). While a dozen fungal isolates were observed in the control, only a single isolate was obtained from the surface-sterilized sample, despite repeated attempts. Next, the subsequent steps of fungal exclusion and purification were followed as per Kusari et al.'s method, with a few modifications in terms of incubation time and use of antibiotics (Kusari et al., 2008).

Morphological study of fungi

The morphological characteristics were recorded on the following parameters: the growth pattern and rate, the colony color, surface texture, marginal shape, and nature of hyphae, using standard manuals (Devi & Prabakaran, 2014). The spores of the endophytes were observed under the microscope (Olympus Biological Microscope Cx23, Japan), described by Sadananda et al (Sadananda et al., 2014).

Molecular identification

A protocol provided by DNeasy Plant Mini Package (QIAgen, USA) was followed for DNA isolation from lyophilized fungal hyphae. The Internal Transcribed Spacer (ITS) regions were amplified and sequenced as described earlier (Azad et al., 2020). After that, the genome sequences were aligned by Chromas software (V 2.6.2) and compared to the nucleotide-nucleotide database BLASTn of NCBI (National Center for Biotechnology Information) (Abarenkov et al., 2010).

Fungal cultivation and extraction

A small-scale cultivation was carried out for each fungal isolate using 40 pairs of Petri dishes and incubated at 28 °C for 21 days. The cultures were frozen to separate water-free contents at average temperatures that were subjected to solvent extraction (ethyl acetate). Then, the filtrated liquid extracts were subjected to rotary evaporation and dried under ambient conditions to eliminate solvent residues (Chowdhury et al., 2016). Subsequently, the concentrated extracts were stored at 4°C.

Determination of fungal bioactivity

Antioxidant Activity

Each 2.0 mL of a 1,1-diphenyl-2-picrylhydrazyl (DPPH) methanol solution (20 µg/mL) was mixed with 2.0 mL of fungal extracts, and a total of ten different final concentrations (200.0 to 0.78125 µg/mL) were prepared. Then, the mixtures were incubated in the dark at room temperature for 30 minutes, and absorbance was measured at 517 nm using a UV spectrophotometer (Brand-Williams et al., 1995). Ascorbic acid was used as a positive control, and 50% inhibitory concentration (IC_{50}) was used to compare the sample values.

Percentage of free radical inhibition: $I\% = 1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}$ x 100

Antimicrobial activity

The disc diffusion method was used to detect antimicrobial activity according to earlier work by Toma & Barriault (1995), with subtle modifications in terms of reference antibiotic. Both bacteria and fungi were taken to measure the sensitivity of three extract

concentrations (80 µg, 40 µg, and 20 µg) per disc: two gram-positive bacteria- *Bacillus megaterium* (ATCC 13578), *Staphylococcus aureus* (ATCC 25923); two gram-negative bacteria- *Escherichia coli* (ATCC 28739), *Pseudomonas aeruginosa* (ATCC 27833), and two fungi- *Aspergillus niger,* and *Aspergillus flavus.* Ciprofloxacin (antibacterial agent) and ketoconazole (antifungal agent) were used as references (30µg/disc) to compare the extracts' activity (Toma & Barriault, 1995).

Cytotoxicity test

The eggs of brine shrimp nauplii (*Artemia salina*) were hatched for 24 hours in saline water. Following Baral et al (2024), ten live brine shrimp nauplii were placed into each of ten test tubes containing 5 mL of salt water, with salinity adjusted to match that of seawater. Test samples were prepared in dimethyl sulfoxide (DMSO) and added to the tubes through serial dilution to achieve concentrations ranging from 100 to 0.19531 µg/mL. For the control group, only 30 µL of DMSO was added to 5 mL of salt water (Baral et al., 2024). The numbers of deaths were counted at different time durations (1 hr, 2 hr, 4 hr, 8 hr, 12 hr, and 14 hr) following the methods mentioned by Hannan et al. (Hannana et al., 2020). Additionally, vincristine sulfate was used as a reference. All tests were triplicated, and logarithmic sample concentration (log[conc.]) was used to determine the 50% lethal concentration (LC_{50}).

Percentage of lethality: $$L\% = \frac{Number\ of\ death\ of\ sample - Number\ of\ death\ of\ Blank}{Number\ of\ death\ of\ Control - Number\ of\ death\ of\ Blank} \times 100$$

Results

Each onion and garlic sample produced one fungus denoted as *A. cepa* endophyte (ACE) and *A. sativum* endophyte (ASE). Although the two fungi exhibited distinct morphological differences, DNA signatures confirmed them both as *Fusarium oxysporum*. Subsequently, their metabolites from small-scale cultivation also showed a significant extent of bioactivity variations. Therefore, the current research describes ACE and ASE as two strains of *F. oxysporum.*

Morphology of the isolates

Both ACE and ASE required approximately 8-10 days to fill Petri dish (9cm diameter) (**Table 1**). Although there is no standard to categorize fungal growth speed, it might be remarked as high speed compared to several fungal growths we have observed during research in a wide array of fungi. Both strains exhibited circular growth pattern, however the speed of colony growth of ASE (8.5 cm/week) was a bit higher than ACE (7.8 cm/week). The most distinguishable feature of the strains was their color. The entire culture period of ACE showed reddish structures from both the top and the bottom of the plates, whereas ASE had minute changes from its vegetative white mycelium to grayish (top) and brownish (bottom) (**Fig 1**). Although the margins of both fungal colonies appeared filamentous, they differed slightly in texture: ACE exhibited a velvety appearance, while ASE was woolly. Additionally, there was a difference in terms of colony side view; ACE was raised, whereas it was flat for ASE (**Table 1**).

Microscopic observation (10× and 100×) revealed the structures of the mycelia and spores. Both fungal isolates exhibited filiform, septate mycelia. The filaments of ACE were very dark and had less diameter compared to ASE (**Fig 1c&1g**). Furthermore, there were spectacular dissimilarities in the shape of their spores. ACE exhibited two forms of fungal micro chlamydospore: some were oval tapering, and some were narrowly amygdaloid. (**Fig 1h**). Nevertheless, very irregularly shaped spores were observed for ACE (**Fig 1d**).

Information on DNA sequencing

BLAST results showed a higher possibility of the two fungi being *F. oxysporum*. The NCBI accession numbers of the fungi are OR062575 and OR062574, isolated from onion and garlic, respectively (**Table 2**).

Bioactivity of fungal extract

Antioxidant activity

The metabolites of both ACE and ASE showed an almost perfect positive correlation between the radicals' inhibition and concentration. The 50% inhibitory concentration (IC_{50}) of reference ascorbic acid was 12.65 µg/ml, whereas ACE and ASE were expressed approximately four to five times. The IC_{50} of ASE (67.46 µg/ml) was nearly one and a half of ACE (45.96 µg/ml) (**Fig. 2**). Thus, the free radicals reducing the capacity of *F. oxysporum* from *A. cepa* exhibited more activity compared to the strain of *A. sativum*.

Antimicrobial activity

Standards ciprofloxacin and ketoconazole showed a zone of inhibition within 35-45 mm against all the respective organisms (**Table 3**). ACE at 80 µg/disc exhibited half of the reference drug (30 µg/disc) activity against *E. coli* (18 mm) and *B. megaterium* (16 mm)*;* one is gram-negative, and the other is gram-positive. On the other hand, ASE showed activity against two gram-negative bacteria, *E. coli* (8 mm) and *P. aurigenosa (*9 mm), at 80 µg/disc dose. Nevertheless, neither strain exhibited any signs of antifungal activity. While ACE showed only antibacterial activity, its effect was markedly stronger than that of ASE.

Cytotoxic activity

The mortality and logarithmic concentration percentages of ACE and ASE were almost perfectly linear. ACE's logarithmic 50% lethal dose (LC_{50}) was 0.44 µg/ml at the 6^{th} hour of sample exposure, which was interestingly lower than the reference LC_{50} of vincristine sulfate (0.57 µg/ml). However, ASE's LC_{50} was five times the reference at 2.55 µg/ml ($p < 0.001$) (**Fig. 3**).

Discussion

The study isolated two endophytic fungi from the flesh of onion and garlic. Molecular data confirmed the fungus *F. oxysporum*, despite differences in morphology, microscopic structures, and bioactivity. This indicates the presence of two distinct strains of the same fungal species in the closely related species *A. cepa* and *A. sativum*.

Research on endophytic fungi typically succeeds in isolating a substantial number of fungal strains in a single attempt. However, this study required two attempts with onion and three with garlic to isolate a single fungus. This difficulty may be attributed to several factors limiting fungal colonization in healthy bulb flesh, such as protective barriers formed by the outer and inner peels, the non-porous waxy cuticle, and chemical defenses against fungal survival (Li et al., 2016; Liu et al., 2019; Yoshida et al., 1987). Nonetheless, the roots could provide a pathway for fungal entry into the aerial parts of these *Allium* species. This hypothesis is supported by the isolation of *F. oxysporum*, a fungus commonly found in the roots of onion and garlic (Nishioka et al., 2019).

F. oxysporum is an asexual soilborne fungus found worldwide in agricultural soil (Arie, 2019; Gordon & Okamoto, 1992). The species is saprophytic, meaning it can grow and live in the rhizospheres of various plants and organic materials in the soil. It enters the vascular system via the roots, causing root rot or tracheomycosis (Garrett, 1970). However, the fungus contains numerous strains that are both pathogenic and nonpathogenic (Sabahi et al., 2022). Interestingly, nonpathogenic strains of *F. oxysporum* are used in biocontrol because their extensive colonization on the root surface outcompetes pathogenic fungi for nutrients (Iida et al., 2022). Our study was conducted on healthy fresh onions and garlic. Since healthy hosts usually carry nonpathogenic endophytes (Trillas & Segarra, 2009), the isolated fungi are unlikely to be pathogenic. To fully address the issue, further study on the pathogenicity of the isolates may be required.

The diverse morphotypes of *F. oxysporum* strains produce asexual septate spore macroconidia of dissimilar lengths and widths (Hafizi et al., 2013). Moreover, colors may vary due to pigments in various strains. Our morphological observations supported this phenomenon: the A. cepa strain produced a red pigment, whereas the A. sativum strain showed no visible pigmentation The onion bulb contains anthocyanins that are responsible for the red or purple color of red onion (Samota et al., 2022), and garlic clove had no such pigments. Despite the lack of evidence for pigment sharing between host plants and endophytic fungi, it is plausible to hypothesize a metabolic interaction between the pigment of the red fungal strain and that of the onion.

In comparative *in vitro* bioactivity analyses, one strain of onion exhibited slightly higher activity than the other strain in antioxidant, antimicrobial, and cytotoxic assays. Several studies on the antioxidant capacity of *F. oxysporum* strains have demonstrated effects on free radical scavenging ability, which are correlated with their polysaccharide content (Caicedo et al., 2019; Chen et al., 2015; Q. X. Wang et al., 2011). The fungal genera contain tremendous antimicrobial and cytotoxic molecules responsible for potential effects (Hyun et al., 2009; Poleto et al., 2021; Sondergaard et al., 2016). An extensive literature review described 185 secondary metabolites from *Fusarium* strains that impart antimicrobial activities, including antibacterial and antifungal (Xu et al., 2023). However, our findings did not indicate any antifungal activity. The current results of the onion strain showed good antimicrobial sensitivity against gram-negative and gram-positive bacteria, and the effect of Garlic's strain was half for gram-negative bacteria. However, previous studies reported that garlic extracts had greater antimicrobial potency than onion extracts, indicating a possible discrepancy (Oyawoye et al., 2022). The most remarkable activity was the cytotoxic activity of the fungus of onion at LC_{50} 0.44 µg/ml, which was lower than the potent antitumor drug vincristine sulfate (LC_{50} 0.57 µg/ml). A recently published work found a reduction in the number of viable cells of Vero cell line on the application of the extract of *F. oxysporum* (Hoque et al., 2022).

Although *F. oxysporum* is a widespread fungus and notoriously recognized in agriculture, no report is available describing the isolation from healthy bulbs and cloves of *Allium*. To the best of our knowledge, this is the first report documenting the isolation of *F. oxysporum* with bioactive potential from healthy garlic cloves and onion bulbs. The parallel investigations on both garlic and onion provided the opportunity to differentiate the fungal strains and keeps some query for further experiments on the causes and scope of the strain variation for agrochemical and pharmacological applications.

Conclusion

This study revealed clear differences between the *F. oxysporum* strains isolated from onion bulbs and garlic cloves. The two strains were distinctly distinguishable based on their growth patterns, as well as macroscopic and microscopic characteristics. Moreover, their extracts showed significant variation in bioactivity, with the onion-derived strain consistently outperforming the garlic-derived strain in all tests.

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Author Contributions

First Author: Perform and Analyzed the data; Second Author: Data Curation and Methodology; Third Author: Perform and Writing- editing; and Fourth Author: Perform, Writing – review; Fifth Author: Supervision, Conceptualization, fund acquisition Writing -review and editing.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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Table 1: Morphology of *Allium cepa* endophyte (ACE) and *Allium sativum* endophyte (ASE) on the culture plate.

Observation of cultures on several duration	ACE	ASE
Growth rate	High (10 days to fill the plate)	High (8 days to fill the plate)
Type of growth	Vegetative up to two weeks	Vegetative up to two weeks
Shape of colony	Circular	Circular
Diameter	7.8 cm at the seventh day	8.5 cm at the seventh day
Color of the top view	Reddish, White circular mycelium in the vegetative stage	White to Gray with age
Color of the bottom view	Reddish, White circular mycelium in the vegetative stage	Grayish to brownish with age
The margin of the colony	Filamentous	Filamentous
Colony surface texture	Velvety	Wooly
Side view of the colony	Raised	Flat

Table 2: Molecular identities of the strains of endophytic fungus *Fusarium oxysporum.*

Features	**ACE**	**ASE**
Base pair number	475 bp	464 bp
NCBI Accession	OR062575	OR062574
Origin	*Allium cepa* bulbs	*Allium sativum* cloves

Table 3: Zone of inhibition of the *Fusarium oxysporum* strains ACE and ASE.

Sample	*E. coli*	*P. aurigenosa*	*B. megaterium*	*S. aureus*	*A. niger*	*A. flavus*
Standards (30 µg/disc)	40 mm	44 mm	35 mm	37 mm	35mm	42 mm
ACE (80, 40 & 20 µg/disc)	18 mm (at 80 µg/disc)	…	16 mm (at 80 µg/disc)	…	…	…
ASE (80, 40 & 20 µg/disc)	8 mm (at 80 µg/disc)	9 mm (at 80 µg/disc)	…	...	...	…

'…' means no activity

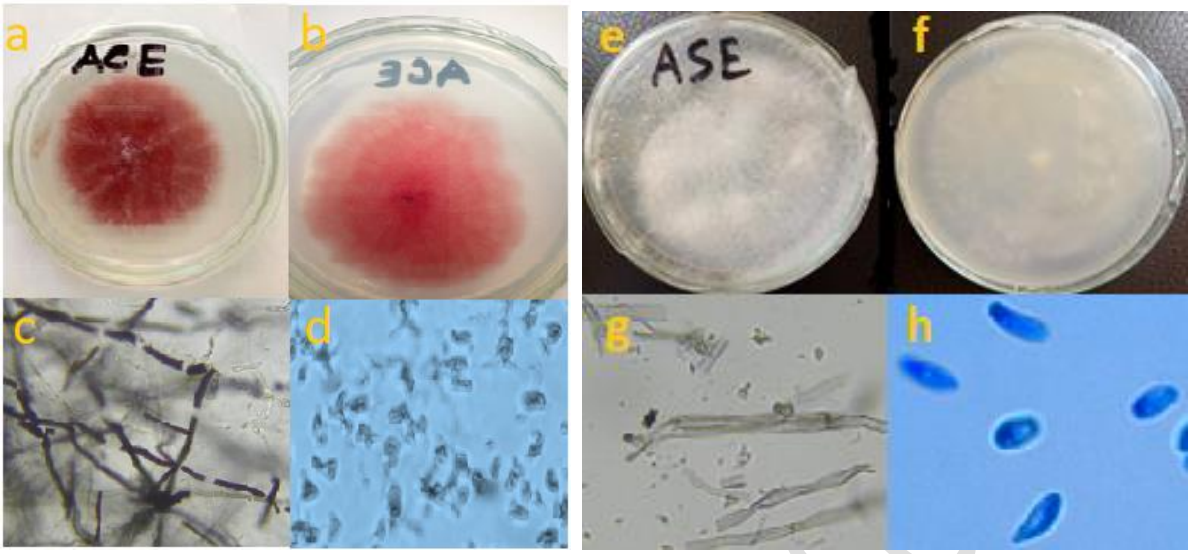
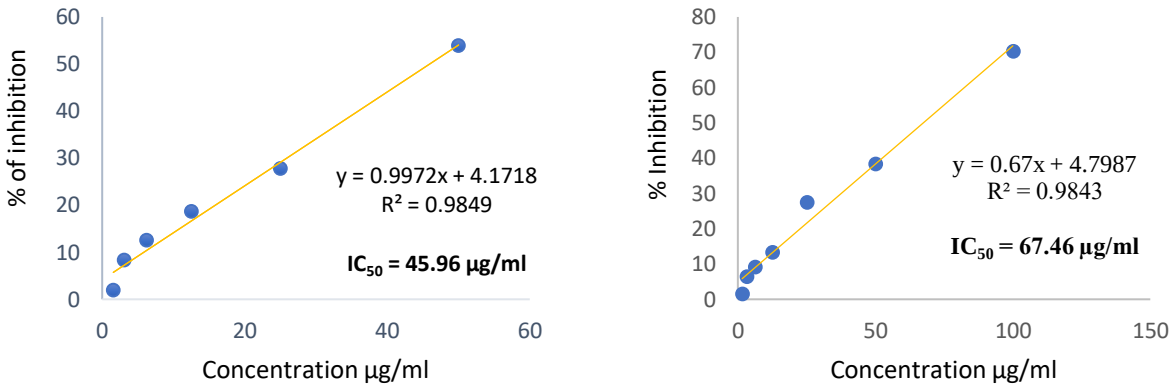


Figure 1: Macroscopic and microscopic observation of *Fusarium oxysporum* strains isolated from onion and garlic. (a-d) strain ACE and (e-h) strain ASE; a & e top view; b & f, bottom view; c (10x) & g (10x) microscopic mycelia; d (10x) & h (100x) microscopic spore.



A **B**

Figure 2: Dose-response relationship on free radical scavenging activity at sixth hour. a and b represent linear regression lines of ACE and ASE, respectively. ACE, *A. cepa* endophyte; ASE, *A. sativum* endophyte.

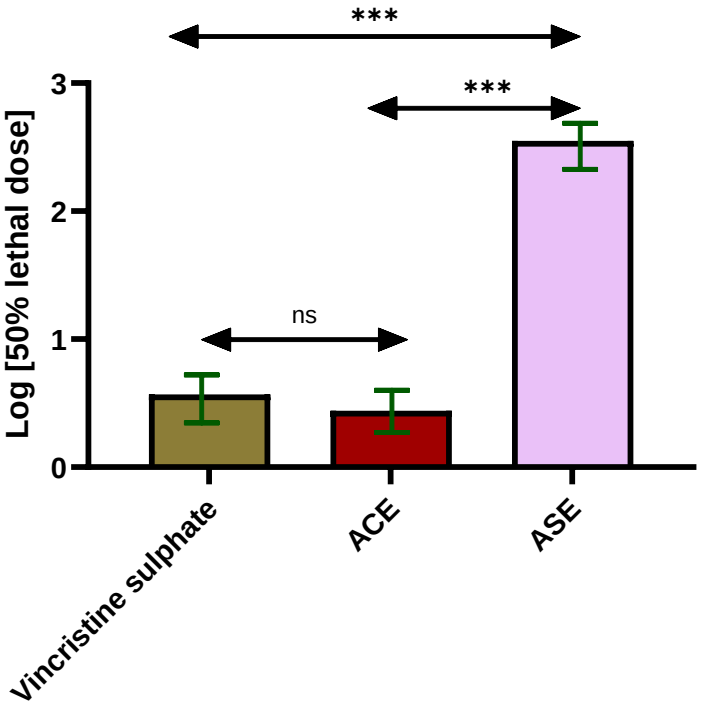


Figure 3: Logarithmic 50% lethal dose (LD_{50}) of sample and standard on brine shrimp nupali after the sixth hour of sample exposure. ACE, *A. cepa* endophyte; ASE, *A. sativum* endophyte. ***, $p < 0.001$; ns, not significant.