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# Determination of percentage growth inhibition of *Fusarium sp.* treated with different solvent extracts obtained through different extraction procedure

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## Abstract

*Fungi are remarkable organisms, known for their ability to synthesize diverse secondary metabolites that play key roles in defence and ecological adaptation. Fungi like Penicillium oxalicum have garnered attention due to their ability for producing secondary metabolites with notable antifungal properties. This study investigated the antifungal activity of Penicillium oxalicum against seven pathogenic Fusarium species, namely F. equiseti, F. poae, F. oxysporum, F. javanicum, F. proliferatum, F. verticillioides, and F. solani. Large-scale production of P. oxalicum was followed by the extraction of bioactive compounds using Soxhlet extraction and column chromatography with a range of polar and non-polar solvents. The antifungal activity of the extracted compounds was assessed using the pour plate method, and growth inhibition of the Fusarium species was recorded. The results demonstrated that solvent selection significantly impacted the antifungal efficacy of both extraction methods. Soxhlet extraction using ethyl acetate exhibited superior inhibition for F. equiseti (57.55%), F. proliferatum (65.73%), and F. javanicum (45.33%). Additionally, hexane in Soxhlet extraction was particularly effective against F. verticillioides (70.28%), while column extraction with isopropanol and ethyl acetate yielded the highest inhibition for F. solani (65.12% and 65.03%, respectively). In some cases, both extraction methods showed similar inhibition rates, as seen with F. poae and F. oxysporum when using ethyl acetate. Overall, ethyl acetate proved to be a highly effective solvent in both extraction methods, particularly in Soxhlet extraction, across multiple Fusarium species. These findings suggested that the extraction method and solvent choice are crucial for the antifungal potential of P. oxalicum metabolites, highlighting their potential use in eco-friendly biocontrol strategies against Fusarium infections in agriculture.*

**Keywords:** Fungi, *Fusarium sp*., *Penicillium oxalicum*, Antifungal activity, Solvent extraction.

## Introduction

Fungi are well known for synthesizing a diverse range of secondary metabolites, many of which exhibit notable biological activities, particularly antifungal effects[1]. Secondary metabolites are organic compounds that do not play a direct role in the primary metabolic functions necessary for the growth, development, or reproduction of fungi[2]. Instead, these compounds often play crucial ecological roles, helping fungi survive in competitive environments by providing defence mechanisms against predators, competitors, and pathogens[3]. Among these metabolites, those with antifungal activity are of particular interest due to their potential in managing plant diseases caused by pathogenic fungi[4].

The production of antifungal secondary metabolites by fungi occurs naturally as part of their ecological interactions. These compounds can inhibit the growth of competing fungi and other microorganisms, ensuring that the producing fungus has access to resources[5]. Many of these bioactive molecules have been isolated and identified as promising candidates for agricultural applications, particularly in the field of biocontrol[6]. For instance, fungal species such as *Trichoderma*, *Penicillium*, and *Aspergillus* have been extensively studied for their ability to produce bioactive metabolites that can suppress the growth of plant pathogens[7].

*Penicillium* species are widely recognized for their antagonistic abilities, allowing them to safeguard host plants from pathogenic fungi by producing bioactive compounds. For instance, *Penicillium oxalicum* has demonstrated effectiveness as a biocontrol agent against numerous fungal diseases in crops, with successful results observed in growth chambers, greenhouses, and field environments[8-10]. *Penicillium citrinum*, isolated from wild banana plantlets, has exhibited significant antagonistic activity against *Fusarium oxysporum*, the pathogen responsible for Fusarium wilt in bananas[11]. Similarly, *Penicillium funiculosum* has been successfully applied to manage bark infections in citrus plants, including lemon trees and orange seedlings, as well as to combat *Phytophthora* root rot in citrus[12]. Additionally, various *Penicillium* species, such as *P. crustosum*, *P. digitatum*, *P. janczewskii*, *P. oxalicum*, and *P. verrucosum*, have proven effective in controlling *Phoma herbarum*, the pathogen responsible for leaf spot disease in mung beans. Among these, *Penicillium janczewskii* displayed the strongest inhibitory effect[13]. These findings underscore the potential of *Penicillium* species as powerful biocontrol agents in

agricultural settings, offering an eco-friendly alternative to chemical fungicides for managing plant diseases.

Research into fungal secondary metabolites has gained momentum in recent years, driven by the need for environmentally sustainable and biologically-based methods of disease control in agriculture. By harnessing the natural antifungal properties of fungi, researchers are exploring ways to mitigate crop losses caused by fungal pathogens, thereby reducing reliance on chemical fungicides and promoting more eco-friendly agricultural practices[14].

## Materials and Methods

**Microorganisms:** This experimental set up was done by selecting a biocontrol fungi *Penicillium oxalicum* and tested against seven numbers of *Fusarium spp.* such as *Fusarium equiseti, Fusarium poae, Fusarium oxysporum, Fusarium javanicum, Fusarium proloferatum, Fusarium verticillioides, Fusarium solani*. These were collected from Microbiology and Plant Pathology Laboratory of Regional Plant Resource Centre, Bhubaneswar, Odisha.

**Mass Scale Production:** Large-scale production of the fungal isolate *Penicillium oxalicum* was initiated by inoculating a 5-day-old culture into 5 Liters of Sabouraud Dextrose medium. This culture was then incubated at 30°C for 12 days. After the incubation period, the mycelial biomass was separated, and the filtrate was collected. Ethyl acetate was added to the filtrate, and the ethyl acetate fraction was evaporated and reserved for the extraction process. Two extraction methods were employed for the secondary metabolites.

**Solvent Extraction Process: Extraction Using Soxhlet Apparatus:** Soxhlet apparatus was employed to extract extracellular secondary metabolites. Both polar and non-polar solvents were utilized for this process. The ethyl acetate-evaporated sample was placed in a thimble within the Soxhlet apparatus, along with a round-bottom flask and a water condenser. A series of solvents, ranging from polar to non-polar (acetic acid, methanol, ethanol, isopropanol, acetonitrile, acetone, chloroform, pyridine, DMSO, ethyl acetate, toluene, and hexane), were used for extraction at temperatures maintained below 100°C[15]. The resulting extracts were evaporated, dissolved in water, and then tested for antifungal activity against seven *Fusarium* species.

**Extraction Using Column Chromatography:** Extracellular secondary metabolites of *Penicillium oxalicum* were further separated using column chromatography. The ethyl acetate-evaporated sample underwent partial purification through a column chromatography process, again using a range of polar to non-polar solvents (acetic acid, methanol, ethanol, isopropanol, acetonitrile, acetone, chloroform, pyridine, DMSO, ethyl acetate, toluene, and hexane)[16]. The resulting extracts were evaporated, dissolved in water, and tested for antifungal activity against seven *Fusarium* species.

**Antifungal Activity:** Antifungal activity of secondary metabolites extracted from *Penicillium oxalicum* using soxhlet extraction and column chromatography method was tested against seven Fusarium spp. Agar plates were prepared by incorporating 100 µl of each solvent extract into molten agar medium. A 6 mm disc containing an actively growing fungal culture (*Fusarium sp.)* was then positioned at the centre of each plate. Colony diameters were measured, and the percentage of growth inhibition was calculated based on the reduction in colony size[17].

## Results and Discussion

This study exhibited the antifungal activity of secondary metabolites extracted from *Penicillium oxalicum* was evaluated using Soxhlet extraction and column chromatography techniques. Various solvent extractions, ranging from polar to non-polar solvents, were tested for their efficacy against seven *Fusarium* species. The antifungal activity of the extracts was assessed in vitro by measuring the percentage inhibition of fungal growth. The study compared the efficiency of Soxhlet extraction (SE) and column extraction (CE) methods across different solvents, highlighting variations in bioactivity against each fungal pathogen.

The results for *Fusarium equiseti* indicated notable differences between the two extraction methods for various solvents. With ethyl acetate, both methods displayed nearly identical inhibition percentages, though SE (57.55±0.58%) showed slightly higher inhibition compared to CE (49.65±1.4%). Chloroform yielded relatively similar results, with CE (41.61±0.4%) performing slightly better than SE (37.59 ±0.70%). Ethyl ether followed a similar trend, with CE (46.29±2.4%) achieving higher inhibition than SE (39.17±2.94%). However, a large contrast was observed with hexane, where SE (49.63±0.51%) showed significantly higher inhibition compared to CE (6.7±1.8%), suggesting that Soxhlet extraction was more effective for hexane. Overall, SE tends to be more effective for most solvents, particularly with ethyl acetate, and hexane, likely due to its exhaustive nature. CE, however, performed better with ethyl acetate and ethyl ether, possibly due to differences in solvent volatility or compound stability during the Soxhlet process (Figure-1).

For *Fusarium poae*, ethyl acetate yielded similar inhibition percentages for both CE (50.36 ± 1.5%) and SE (50.42 ± 0.60%). However, with acetone, CE demonstrated much higher inhibition (49.61 ± 0.5%) compared to SE (20.45 ± 2.01%), indicating greater efficiency of CE for this solvent. Chloroform results were comparable for both methods, with CE (45.185 ± 1.52%) slightly outperforming SE (43.185 ± 0.80%). Ethyl ether followed a similar pattern, with CE (48.885 ± 0.51%) and SE (47.545 ± 3.47%) showing close results. These findings suggest that, both methods were efficient for ethyl acetate, for extraction of bioactive antifungal compounds (Figure-2).

Against *Fusarium oxysporum*, CE exhibited a slightly higher inhibition with ethyl acetate (46.88 ± 0.8%) compared to SE (42.84 ± 2.48%). Conversely, SE showed better results with chloroform (36.60 ± 6.08%) compared to CE (30.62 ± 0.88%). Ethyl ether also favoured SE (38.17 ± 0.41%) over CE (25.63 ± 0.88%). These variations indicate that CE is more effective with ethyl acetate, while SE is preferable for chloroform and ethyl ether (Figure–3).

For *Fusarium javanicum*, the use of ethyl acetate resulted in a slight advantage for CE (47.01 ± 0.49%) over SE (45.34 ± 0.47%). However, with chloroform, CE exhibited significantly higher inhibition (42.38±0.39%) than SE (16.80±2.41%), indicating that CE has superior efficiency with this solvent. A similar trend was observed with ethyl ether, where CE (39.73 ± 0.37%) outperformed SE (22.43 ± 0.30%). Hexane showed an inverse pattern, with SE (43.68±1.86%) exhibiting considerable inhibition, while CE showed no inhibition (0%). This comparison highlights the effectiveness of SE for hexane, while CE was more efficient with ethyl acetate, chloroform, and ethyl ether (Figure – 4).

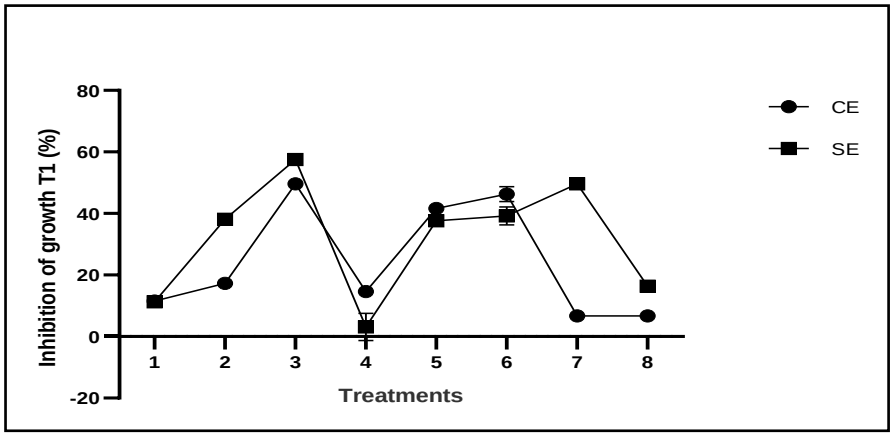


**Figure-1:** Effect of solvent extracts of *P. oxalicum* culture against *F. equiseti.*

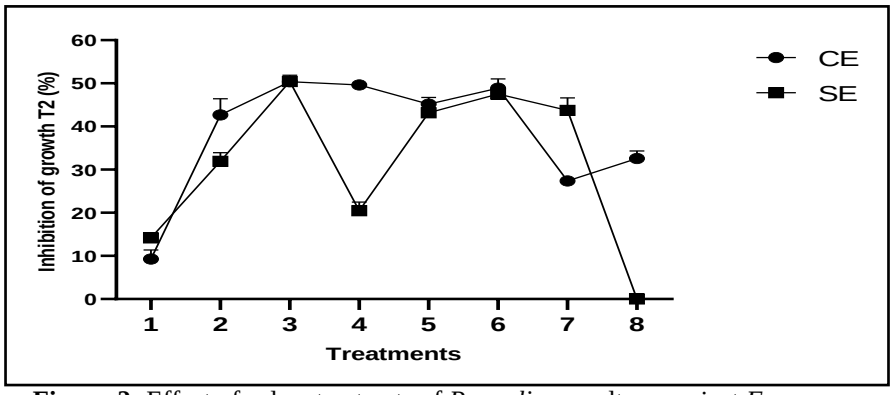


**Figure-2:** Effect of solvent extracts of *P. oxalicum* culture against *F. poae.*

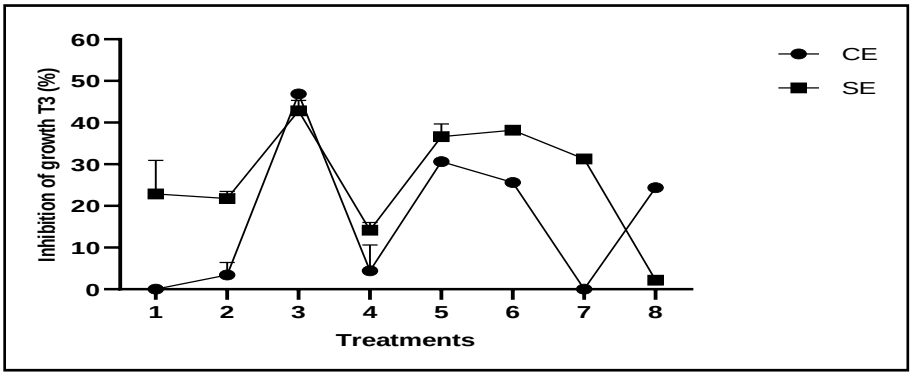


**Figure-3:** Effect of solvent extracts of *P. oxalicum* culture against *F. oxysporum.*

The results for *Fusarium proliferatum* demonstrated distinct differences between the two methods. SE exhibited significantly higher inhibition with isopropanol (53.145 ± 0.53%) compared to CE (23.75 ± 3.29%). Similarly, with ethyl acetate, SE (65.73 ±0.65%) outperformed CE (48.92±2.51%). However, for chloroform, CE (41.83 ± 0.58%) was much more effective than SE (14.84 ± 1.18%). Ethyl ether followed the same trend, with CE (41.25 ± 1.76%) showing higher inhibition than SE (19.80 ± 0.28%). Hexane once again demonstrated the superiority with SE (56.62 ± 2.4%) showing much higher inhibition as compared to CE (0%). These results suggest that while SE is more effective with isopropanol, ethyl acetate, and hexane, CE is better suited for ethyl acetate, chloroform and ethyl ether (Figure–5).

In the case of *Fusarium solani*, CE (65.12 ± 6.5%) showed slightly higher inhibition with isopropanol compared to SE (58.49±1.35%), though both methods displayed strong antifungal activity. SE, however, outperformed CE with ethyl acetate (72.1 ± 1.23% vs. 65.03 ± 0.5%) and hexane (68.02 ± 0.53% vs. 1.81 ± 0.82%). For chloroform and ethyl ether, CE (58.88 ± 1.22% and 54.59 ± 0.38%) was more effective than SE (20.50 ± 6.36% and 13.69 ± 2.39%, respectively). These results further emphasize that the efficacy of the extraction method varies with the solvent, with SE being more suitable for ethyl acetate and hexane, while CE is more efficient for chloroform and ethyl ether (Figure-7).

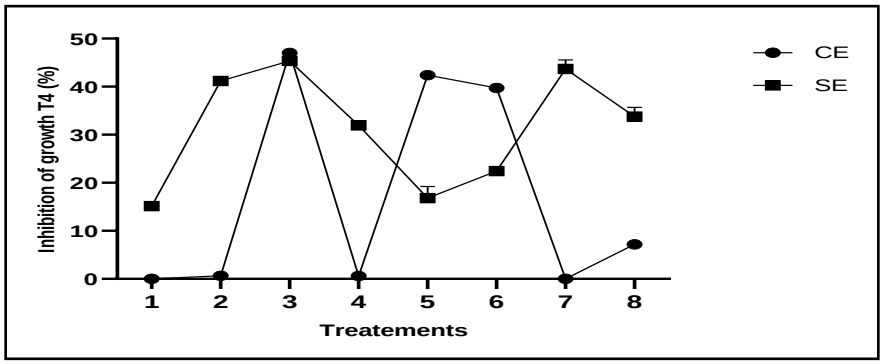


**Figure-4:** Effect of solvent extracts of *P. oxalicum* culture against *F. javanicum.*

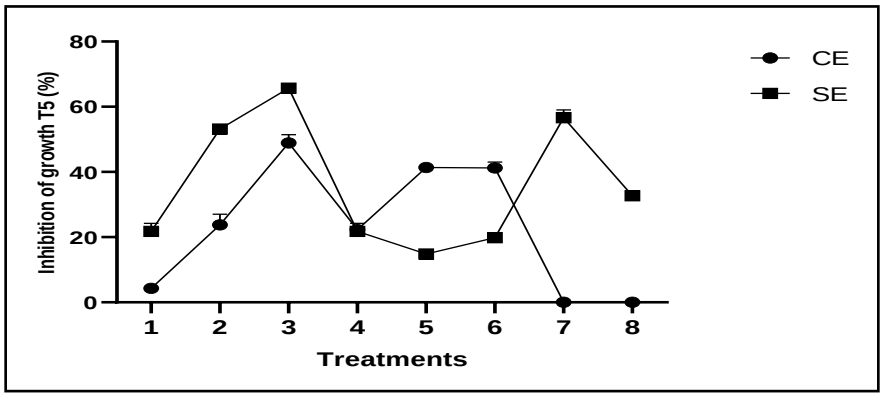


**Figure-5:** Effect of solvent extracts of *P. oxalicum* culture against *F. proliferatum.*

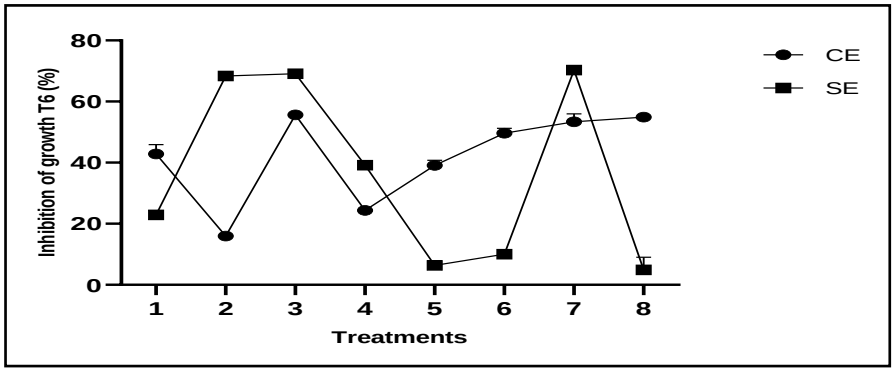


**Figure-6:** Effect of solvent extracts of *P. oxalicum* culture against *F. verticillioides.*

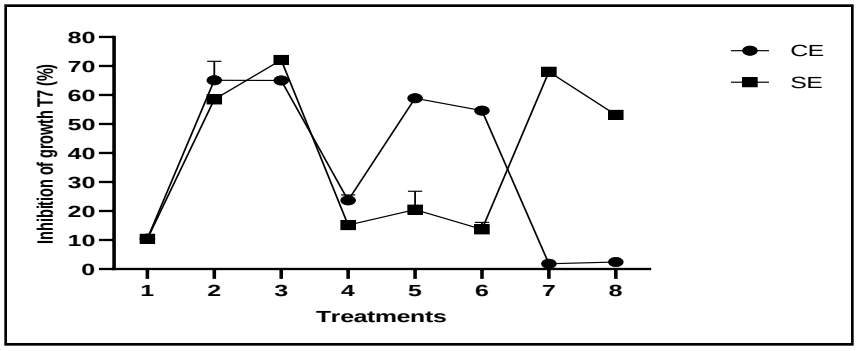


**Figure-7:** Effect of solvent extracts of *P. oxalicum* culture against *F. solani.*

Overall, this study highlights the importance of selecting the appropriate extraction method based on the solvent used, as the efficiency in isolating antifungal compounds varies considerably between Soxhlet and column extraction methods.

**Discussion:** The comparison between column extraction (CE) and Soxhlet extraction (SE) methods using different solvents revealed distinct variations in antifungal efficacy, demonstrating the critical role of solvent selection in optimizing the inhibition of Fusarium species. Against *Fusarium equiseti*, Soxhlet extraction using ethyl acetate exhibited a significantly higher inhibition rate (57.55 ± 0.58%) compared to column extraction (49.65 ± 1.4%). This suggests that Soxhlet extraction might be more effective for extracting bioactive compounds from ethyl acetate in inhibiting *F. equiseti*. For *Fusarium poae*, both methods yielded comparable results with ethyl acetate (CE: 50.36 ± 1.5%, SE: 50.42 ± 0.60%), indicating that ethyl acetate is a suitable solvent for either extraction method when targeting this species. In the case of *Fusarium oxysporum*, CE and SE with ethyl acetate also showed relatively similar inhibition rates (CE: 46.88 ± 0.8%, SE: 42.84 ± 2.47%), suggesting that this solvent might be universally effective against *F. oxysporum*. Interestingly, *Fusarium javanicum* was more susceptible to inhibition through Soxhlet extraction with ethyl acetate (45.33 ± 0.4%). The comparison of extraction methods for *Fusarium proliferatum* revealed that Soxhlet extraction with ethyl acetate (65.73 ± 0.65%) was significantly more effective than column extraction (48.92 ± 2.51%), which aligns with previous findings that ethyl acetate is an effective solvent for Soxhlet extraction across multiple Fusarium species. In the case of *Fusarium verticillioides*, SE with hexane (70.28 ± 1.018%) exhibited the highest inhibition, while CE performed better with ethyl acetate (55.64 ± 0.59%). For *Fusarium solani*, CE displayed stronger inhibition with isopropanol (65.12 ± 6.5%) and ethyl acetate (65.03 ± 0.5%), whereas Soxhlet extraction with ethyl acetate showed superior inhibition (72.10 ± 1.23%). These results again highlight ethyl acetate's efficiency in SE across various Fusarium species, but also underline the effectiveness of polar solvents like isopropanol when using column extraction.

Several studies support these findings, emphasizing the significance of solvent and method selection in optimizing antifungal activity. For instance, ethyl acetate extracts of *Trichoderma* spp. through column chromatography showed biocontrol properties by reducing the mycelial growth of *Fusarium oxysporum*[18]. This highlights that ethyl acetate can effectively extract bioactive metabolites that inhibit *Fusarium spp*. In another study, ethanolic extracts (70%) from a liquid culture of *T. longibrachiatum* also showed significant antifungal activity against *F. oxysporum* through mycelial growth inhibition assays, further supporting the role of effective solvents in antifungal strategies[19]. Moreover, *Epicocum nigrum* isolated from *Euphorbia milii* leaves demonstrated high antifungal activity against *F. solani* in dual culture tests, with ethyl acetate extracts proving highly effective in inhibiting *F. solani*[20]. This finding mirrors the observation that ethyl acetate extracts are highly potent against *F. solani*, regardless of the extraction method used. The antagonistic activity of endophytic fungi such as *Aspergillus* spp., isolated from *Plectranthus amboinicus* leaves, further demonstrated the biocontrol potential of these fungi against *Fusarium verticillioides*[21]. Similarly, *Alternaria alternata* and *Trichoderma longibrachiatum* demonstrated antagonistic activity against *F. oxysporum* and *F. proliferatum* in a dual culture assay, which aligns with the efficacy of these fungi in suppressing *Fusarium* species[22]. The effectiveness of *Trichoderma harzianum* and *Alternaria infectoria* as biocontrol agents against *Fusarium* species, such as *F. equiseti*, was further demonstrated in a study focusing on wheat crown rot control[23].

Therefore, ethyl acetate consistently demonstrated its potency as a solvent for the extraction of bioactive antifungal compounds in both Soxhlet and column extraction methods. Its capacity to inhibit the growth of various *Fusarium* species, especially when using Soxhlet extraction, highlights its importance in obtaining secondary metabolites with strong biocontrol potential. This study highlights the importance of selecting ethyl acetate for solvent-based extractions, especially when employing Soxhlet extraction, to induce antifungal efficacy across a range of fungal pathogens.

## Conclusion

This study highlights the importance of solvent selection and extraction methods in optimizing the antifungal activity of secondary metabolites against various *Fusarium* species. Ethyl

acetate consistently demonstrated high efficacy, particularly in Soxhlet extraction, making it a valuable solvent for isolating bioactive compounds. However, the study also shows that non-polar solvents like chloroform, hexane, and isopropanol can be effective depending on the species and extraction method. The findings are supported by other studies, reinforcing the importance of solvent selection in the development of biocontrol strategies. Future research should focus on refining solvent and method combinations to improve antifungal activity and explore the potential for integrating these findings into practical agricultural applications for disease control.

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