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Research Article

# EVALUATION OF ANTIFUNGAL POTENTIAL OF *ALLIUM AMPELOPRASUM* AGAINST FUNGAL PHYTOPATHOGENS

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**ABSTRACT**

**Objective:** Present study evaluates the antifungal potential of *Allium ampeloprasum* extracts against two phytopathogens of economic importance, namely *Alternaria triticina* and *Magnaporthe oryzae* which are found to be one of the greatest threats to wheat and rice crop worldwide.

**Methods:** Four different solvents, i.e., water, methanol, acetone, and hexane were used for extract preparation based on the increasing order of polarity. Further, different concentrations of these extracts were used to perform a quantitative assay for measuring the antifungal activity of extracts. Two-way analysis of variance was used to statistically analyze the results through GraphPad Prism v. 7.00.

**Results:** The best results were shown by water extracts with a maximum inhibition of 99.39±0.2% against *A. triticina* and 99.39±2.2% against *M. oryzae*. Water extracts gave minimal inhibitory concentration 90 values at 9.94 µg/mL against *M. oryzae* and at 16.73 µg/mL against *A. triticina*.

**Conclusion:** Water extracts of *A. ampeloprasum* have relatively higher potential for being used as an antifungal agent as compared to other organic extracts used in this study. Further study on the chemical composition of *A. ampeloprasum* water extracts can reveal potent anti-phytopathogenic fungal compounds which can be used to develop biofungicides.

**Keywords:** *Allium ampeloprasum*, *Alternaria*, *Magnaporthe*, Antifungal, Natural compounds.

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## INTRODUCTION

Fungicides play a significant role in improving crop production and stability, and synthetic fungicides are extensively used worldwide despite their hazards outweigh their benefits. Some fungicides strongly bound to soil instigating soil contamination which may decline mycorrhizal fungi population in soil affecting soil fertility and may also cause groundwater contamination. It is found that the consumption rate of fungicides in India is 13% and is 21% at world level [1]. Natural plant products provide limitless prospects for new bioactive compounds both as crude extracts and as pure compounds [2]. Many natural products obtained from plants having biocidal activity are considered as an alternate effective and eco-friendly source against phytopathogens [3]. Moreover, these natural products are usually cheaper than commercial fungicides and are easily accessible to farmers [4]. *Allium* species is recognized as an important genus of the Alliaceae family and well known for its biocidal properties [5]. Earlier studies have reported that allicin, ajoene, thiosulfinates, and few other phenolic and nitrogen-based bioactive compounds are responsible for the biocidal activity of *Allium* species [6,7]. *Allium ampeloprasum* has been considered more beneficial than other *Allium* species [8] and is known to exhibit strong antifungal activity like *A. sativum* [9]. A comparative evaluation of the ethanol extracts of *A. sativum* and *A. ampeloprasum* showed that *A. ampeloprasum* possess better antifungal activity and its mild odor makes it preferable for its use as agricultural fungicide [10].

The present study is conducted to examine the antifungal potential of the clove and residue of *A. ampeloprasum* using different organic solvents against two phytopathogens, namely *Alternaria triticina* and *Magnaporthe oryzae*. These phytopathogens are threat to crop safety throughout the world; wherein *A. triticina* is India's second most prevalent wheat pathogen [11] while *M. oryzae* is a rice pathogen which is claimed to be world's most economically harmful phytopathogen [12].

## METHODS

### Plant material and chemicals

Fresh sample of *A. ampeloprasum* was obtained from Dosanjh Agricultural Research and Development Farm, Punjab, India. Organic solvents hexane, acetone and methanol were procured from Loba Chemie, and media preparations potato dextrose agar (PDA) and potato dextrose broth (PDB) were procured from HiMedia.

### Preparation of extracts

The *A. ampeloprasum* sample was thoroughly washed with tap water followed by distilled water to remove any impurities. The cloves were separated from rest of the bulb and allowed to dry in a sterile environment at room temperature; similar treatment was given to the residue. Extraction process as described by Brusotti *et al.* [13] was used to prepare the extracts using organic solvents in increasing order of their polarity, namely hexane, acetone, methanol, and water with polarity of 0.009, 0.355, 0.762, and 1.000, respectively. Further, the cloves were weighed, crushed in mortar and pestle and then immersed in hexane in 1:1 (w/v) ratio and incubated at room temperature for 48 h with intermittent stirring to allow maximum diffusion of extracts in solvent. Solvent containing extract was filtered by Whatman filter paper No. 1 and collected in a separate conical flask. The residual was then immersed in acetone for further extraction using a similar procedure and subsequently in other solvents based on the increasing order of their polarity [14]. Likewise, preparation of an extract from *A. ampeloprasum* residue was also accomplished; *A. ampeloprasum* residue was comprised the peel, stalk, clove skin, stem, basal plate, and root of the bulb. Solvents containing the extracts were concentrated at 50°C to 55°C using a vacuum evaporator followed by further drying at room temperature in Petri plate to obtain the powdered extracts. The extracts were then dissolved in their respective solvents to attain a concentration of 1000 mg/mL (w/v), sterilized using 0.22-micron filters and the active constituents from clove and residue of *A. ampeloprasum*, a

total of 8 different extracts were obtained which were further subjected to antifungal testing.

**Fungal strains and isolation of spores**

The fungal strains used in this study, namely *A. triticina* (Indian Type Culture Collection [ITCC 5496]) and *M. oryzae* (ITCC 6808) were attained as pure cultures from the ITCC, New Delhi, India. The cultures were revived on PDA at 27°C for 5–10 days using plates and slants; the fungal cultures were renewed after every 15–20 days to keep the culture fresh. Spores were harvested as per the protocol used by Broekart *et al*. [15], the Petri plates were flooded with PDB, and a sterile spatula was used to detach the spores of the mycelium. The spores were filtered through Whatman filter paper No. 1 and then centrifuged. A spore count of $2*10^8$ was maintained in PDB which was calculated using Neubauer's chamber, and then the fungal spore suspension (spores in PDB) was stored in 50% glycerol at −20°C for further use.

**Testing of antifungal potential**

A 96-well microtiter plate was used to measure the fungal growth inhibition. 200 µL *A. ampeloprasum* extract with a concentration of 200 µg/mL was added to each well of series A and the remaining plate was filled with 100 µL of PDB. The serial dilution was done by adding 100 µL of *A. ampeloprasum* extract from series A to series B and then from series B to series C, and so on until series G. The remaining 100 µL from series G was discarded, and series H was filled with 100 µL pf PDB only and was treated as control. Different plates were used for different extracts and fungi to prevent cross-contamination between the results. The fungal spore suspension was thawed and diluted to make the spore count of $2*10^6$, and 100 µL of fungal spore suspension was added to each well of column 1, 2, and 3. The column 4, 5, and 6 was filled with PDB (100 µL) referred as test blank. The final concentration of extracts in each well following the addition of 100 µL of fungal spore suspension or 100 µL PDB in respective wells, was reduced to half of their initial concentration. Therefore, the final *A. ampeloprasum* extracts concentration ranging from 100 µg/ml to 1.5625 µg/mL from series A to G was obtained. The wells 1, 2, and 3 in series H were filled with 100 µL of PDB and referred to as negative control, while the well 4, 5, and 6 were referred to as positive control since they contain 100 µL of PDB and 100 µL of fungal spore suspension. The titer plates were incubated at 27°C for 48 h and to allow sufficient growth and the readings were measured through microplate reader (Bio-Rad at 595 nm).

**Minimum inhibitory concentration (MIC)**

The MIC was considered as the concentration at which 50% or 90% of growth was inhibited, thus $MIC_{50}$ and $MIC_{90}$. The percentage of growth inhibition was measured using the equation; $\left[\frac{(\Delta C - \Delta T)}{\Delta C}\right]*100$, where, ΔC is the difference between the average absorbance of positive control and negative control, ΔT is the difference between the average absorbance of test and test blank [16].

**Statistical analysis**

Percentage growth inhibition was presented as a mean ± standard deviation, and MIC, i.e., $MIC_{50}$ or $MIC_{90}$ values were determined by the software, Graph v. 4.4.2. The data were statistically analyzed by means of regular two-way analysis of variance at 95% confidence interval by the software, GraphPad Prism v. 7.00 and Bonferroni post-test was used to compare the percentage growth inhibition of water extracts to other extracts.

**RESULTS**

Comparative evaluation of the clove and residue extracts reveals that the antifungal activity of clove extracts increases with an increase in polarity of solvents while the antifungal activity of residue extracts is higher in less polar solvents like hexane. The results of the percentage growth inhibition observed in phytopathogens against different crude extracts of *A. ampeloprasum* clove and residue have been expressed in Tables 1 and 2, respectively. The water extract of *A. ampeloprasum* clove indicated highest antifungal activity among all the tested extracts with maximum fungal growth inhibition of 99.39% against *A. triticina and M. oryzae*. However, the concentration of extract differed in each case; *A. triticina* showed 99.39±0.2% growth inhibition at extract concentration of 100 µg/mL while *M. oryzae* showed 99.39±2.2% growth inhibition at extract concentration of 12.25 µg/mL. Moreover, the acetone extract of *A. ampeloprasum* clove with maximum growth inhibition of 98.68±1.1% at 3.125 µg/mL and the hexane extract of *A. ampeloprasum* residue with maximum growth inhibition of 83.12±1.2% at 100 µg/mL against *M. oryzae* were noted as second and third best antifungal extracts, respectively.

Furthermore, some extracts showed poor antifungal activity while others promoted fungal growth.

For instance, among the *A. ampeloprasum* clove extracts, hexane and methanol extracts showed poor growth inhibition of *A. triticina*, and acetone extracts slightly promoted its growth at low extract concentrations. Likewise, hexane and methanol extracts of *A. ampeloprasum* clove also poorly inhibited the growth of *M. oryzae*, while promoting its growth at certain concentrations as described in Fig. 1a. Conversely, among the *A. ampeloprasum* residue extracts water, methanol and acetone extracts showed poor antifungal activity against both the fungi as shown in Figs. 2b and 2b. Clove water extract

**Table 1: Percentage growth inhibition of *A. triticina* against different crude extracts**

| Concentration of extracts (in µg/mL) | Hexane | Acetone | Methanol | Water |
|---|---|---|---|---|
| Clove | | | | |
| 100.00 | 21.63±0.7*** | 1.16±0.3*** | 25.69±1.4*** | 99.39±0.2 |
| 50.00 | 29.18±0.4*** | 3.68±0.4*** | 19.44±0.9*** | 98.69±0.5 |
| 25.00 | 17.34±0.8*** | 11.23±1.3*** | 20.75±1.1*** | 88.58±1.6 |
| 12.50 | 16.08±0.1*** | −3.22±0.4*** | 25.59±1.5*** | 83.08±0.8 |
| 6.25 | 3.26±0.4*** | −10.49±1.5*** | 23.59±2.3*** | 83.50±0.8 |
| 3.125 | 5.27±0.6*** | −3.50±0.6*** | 27.79±0.4*** | 75.43±1.7 |
| 1.5625 | 3.49±1.9*** | −7.55±1.5*** | 20.23±3.7*** | 83.03±0.4 |
| Residue | | | | |
| 100.00 | 34.27±0.4*** | 13.24±1.8*** | 19.07±1.6*** | 22.28±0.9*** |
| 50.00 | 29.56±0.5*** | 14.55±0.6*** | 13.71±1.0*** | 20.75±0.5*** |
| 25.00 | 30.91±0.2*** | 17.20±0.5*** | 31.70±0.3*** | 19.53±1.3*** |
| 12.50 | 25.64±0.8*** | 8.95±0.3*** | 31.42±1.8*** | 16.74±1.5*** |
| 6.25 | 23.45±0.2*** | 8.58±0.7*** | 30.26±0.1*** | −18.83±1.5*** |
| 3.125 | 19.77±0.3*** | 0.65±0.4*** | 30.12±0.9*** | −15.71±1.4*** |
| 1.5625 | 25.92±0.1*** | 1.96±0.6*** | 36.83±0.4*** | −15.90±1.6*** |

***Indicates (p<0.001), **indicates (p<0.01), ns: Indicates (p>0.05). *A. triticina: Alternaria triticina*

**Table 2: Percentage growth inhibition of *M. oryzae* against different crude extracts**

| Concentration of extracts (in µg/mL) | Hexane | Acetone | Methanol | Water |
|---|---|---|---|---|
| Clove | | | | |
| 100.00 | −37.86±1.3*** | 61.23±0.9*** | −3.25±1.0*** | 50.58±5.5 |
| 50.00 | −37.21±1.0*** | 76.63±0.9*** | −22.13±1.1*** | 36.08±2.3 |
| 25.00 | −56.83±3.2*** | 86.29±1.2ns | −20.39±1.4*** | 82.14±1.5 |
| 12.50 | 10.78±1.0*** | 92.39±1.4*** | 1.95±2.3*** | 99.39±2.2 |
| 6.25 | 13.08±1.5*** | 88.02±1.4*** | −1.63±1.2*** | 76.47±1.9 |
| 3.125 | 29.09±0.5*** | 98.68±1.1*** | 10.30±1.2*** | 61.87±1.4 |
| 1.5625 | 55.60±2.2*** | 79.64±1.1** | −33.19±0.9*** | 86.05±0.7 |
| Residue | | | | |
| 100.00 | 83.12±1.2*** | 15.49±0.3*** | 10.41±0.9*** | 48.37±2.6ns |
| 50.00 | 78.02±1.3*** | 29.34±1.7*** | −6.18±3.4*** | 40.14±6.0ns |
| 25.00 | 45.62±1.3*** | 25.97±1.3*** | −34.38±0.6*** | 26.69±0.5*** |
| 12.50 | 40.45±1.3*** | 15.03±3.9*** | −21.37±1.4*** | 8.63±4.7*** |
| 6.25 | 27.87±1.3*** | 7.29±1.1*** | −20.82±1.0*** | 34.87±2.4*** |
| 3.125 | 21.26±1.6*** | −3.05±1.0*** | −74.40±1.1*** | 1.15±1.0*** |
| 1.5625 | 23.49±0.9*** | −9.02±1.7*** | −84.38±1.7*** | −12.49±2.6*** |

***Indicates (p<0.001), **indicates (p<0.01), ns: Indicates (p>0.05). *M. oryzae: Magnaporthe oryzae*

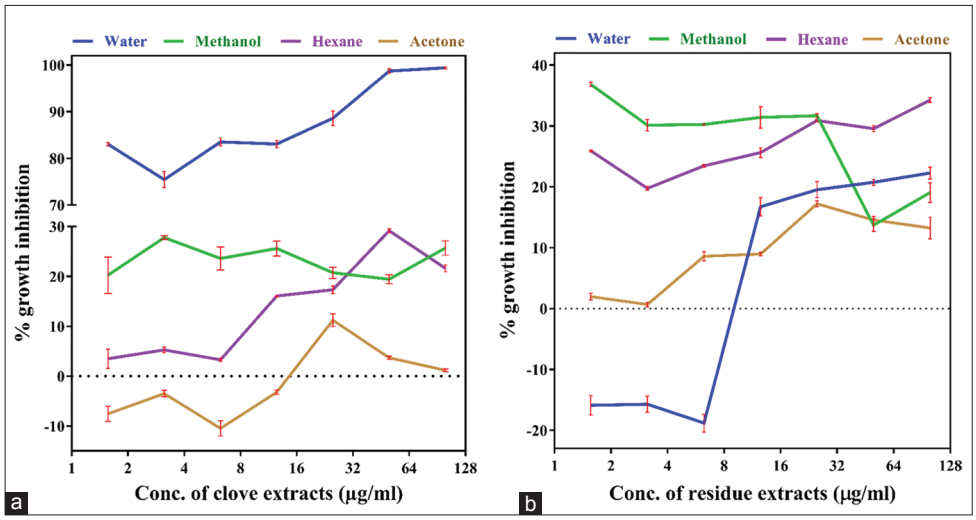


**Fig. 1: Effect of the *Allium ampeloprasum* crude extracts on the growth of *Alternaria triticina* (a) clove extracts (b) residue extracts**

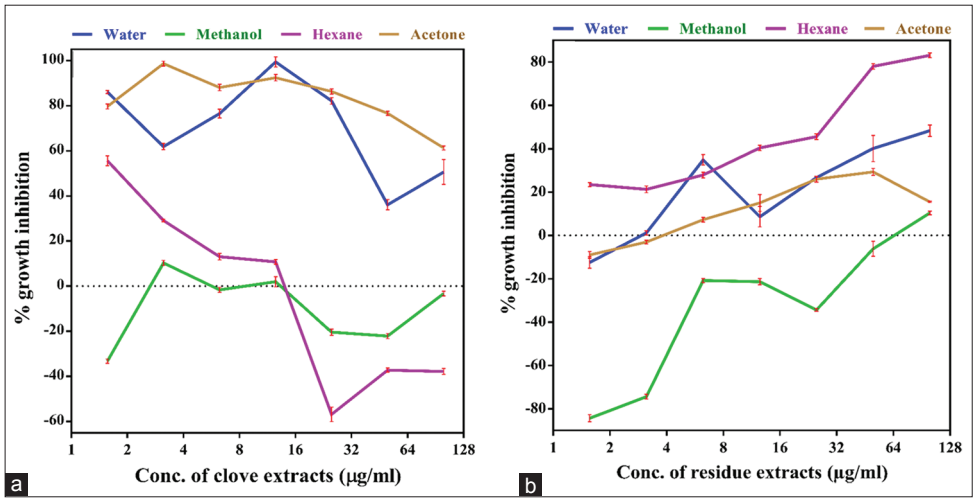


**Fig. 2: Effect of the *Allium ampeloprasum* crude organic extracts on the growth of *Magnaporthe oryzae* (a) clove extracts (b) residue extracts**

promoted the growth of *A. triticina* at lower concentrations, as shown in Fig. 2a. While acetone, methanol and water clove extracts promoted the growth of *M. oryzae* at various concentrations, the details are discussed in Table 2.

A total of 56 different combinations of extracts were tested against each fungus, on considering the extract from two sources, four organic solvents, and seven different concentrations as individual extracts. Extracts showing more than 25% growth inhibition were considered

significant; according to which 37.50% extracts showed significant growth inhibition against *A. triticina,* i.e., 21 of 56 extracts and 48.21% showed significant growth inhibition against *M. oryzae,* i.e., 27 of 56 extracts. On the contrary, the extracts showing negative growth inhibition were basically promoting fungal growth; the analysis of results shows that 12.50%, 7 extracts among the total of 56 extracts promoted growth of *A. triticina* while 30.35% extracts, i.e., 17 of 56 extracts promoted growth of *M. oryzae*.

MIC, namely $MIC_{50}$ and $MIC_{90}$ values were calculated to evaluate the antifungal potential of extracts, and the details are displayed in Fig. 3. In case of the water extract of *A. ampeloprasum* clove, $MIC_{50}$ value was obtained at 42.24 µg/mL concentration, and $MIC_{90}$ value was obtained at 9.94 µg/mL concentration against *M. oryzae*, whereas only $MIC_{90}$ value was observed at 16.73 µg/mL concentration against *A. triticina*. In addition, the *A. ampeloprasum* clove extracts showed the $MIC_{90}$ value of 2.41 µg/mL and $MIC_{50}$ value of 1.89 µg/mL against M. oryzae for acetone and hexane extract, respectively. Whereas, among the *A. ampeloprasum* residue extracts $MIC_{50}$ value at 28.32 µg/mL was shown by hexane extract against *M. oryzae.* Based on this information, it could be established that the water extract of *A. ampeloprasum* clove has the best antifungal potential for further use as an antifungal agent. Therefore, the water extracts were used as standards for statistical analysis of all extracts and the results shows that all the extracts. Results indicate that all extracts were significantly different against *A. triticina* with $p<0.001$, while in the case of *M. oryzae* all but four extracts were significantly different with $p<0.001$.

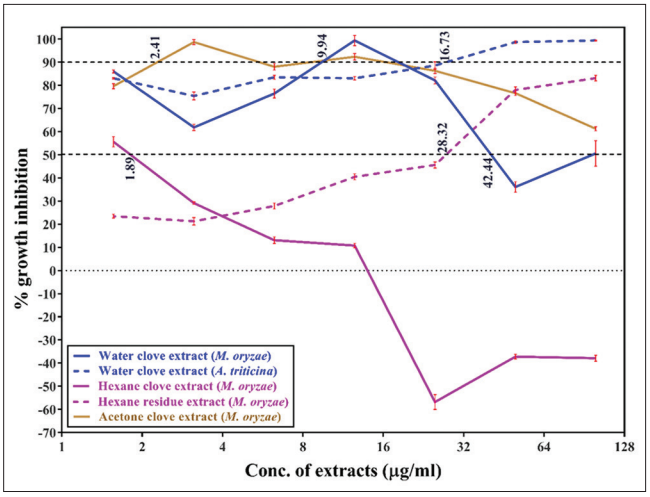


**Fig. 3: Minimum inhibitory concentration$_{50}$ ($MIC_{50}$) and $MIC_{90}$ values of the organic extracts in different solvents**

## DISCUSSION

*Allium* species are known to inhibit a wide range of pathogenic fungi mostly due to the presence of allicin, ajoene, and organosulfur compounds [17-19]. Analysis of 29 different plant extracts against *Alternaria* species shows that 31%, nine plant extracts exhibit significant antifungal activity where *Cynara scolymus*, *Lippia alba*, *Salvia sclarea*, and *Salvia officinalis* showed approximately 98% growth inhibition of *Alternaria* species which is equivalent to the growth inhibition shown by commercial fungicides [16]. A study conducted on pathogen infested wheat suggests that coating wheat seedlings with *Allium* juice before sowing leads to better growth and advocates fungicidal potential of *A. ampeloprasum* products [20].

Antifungal analysis of *A. sativum* extracts against different fungal pathogens of coffee tree shows that the *A. ampeloprasum* extracts can inhibit mycelial growth by 100% as well as also effectively preventing the germination of spores [4]. It is found that the mechanism of action, of *A. ampeloprasum* as an antifungal agent, is due to the competitive inhibition of sulfhydryl-containing enzymes by allicin and the inhibition of lipid biosynthesis leading to cell wall damage, as seen in the case of *Candida albicans* [9,21]. In accordance to our study, a former study also concludes that elephant *A. ampeloprasum* clove has better activity than *A. ampeloprasum* skin and seed [9]. The efficacy of cinnamic acid derivatives isolated from *A. ampeloprasum* against four fungal pathogens and cheap traceability makes them a potential fungicide [6]. Three steroidal saponins and aginoside isolated from *A. ampeloprasum* were found to exhibit antifungal activity against *Mortierella ramanniana* [22]. An *in vitro* study on biologically active saponin isolated from seeds *A. ampeloprasum* reveals that butanolic extracts were found to completely inhibit the growth of *Alternaria alternata* at 200 µg/mL concentration and a concentration of 400 µg/mL, the butanolic extracts completely inhibits the growth of *Fusarium roseum*, *Colletotrichium dematium*, *Curvularia lunata*, and *Aspergillus fumigatus* [23]. A recent study on ethanolic extracts obtained from *Allium* species supports that *A. ampeloprasum* possesses antifungal potentially against *A. triticina* and *M. oryzae* [10].

## CONCLUSION

All the crude extracts of *A. ampeloprasum* clove and *A. ampeloprasum* residue showed erratic antifungal activity irrespective of extract's concentration. Clove extract showed much better antifungal activity than residue extracts. Acetone extracts showed the weakest activity against *A. triticina,* and methanol extract showed the weakest activity against *M. oryzae*. It could be concluded from this study that, water extract of *A. ampeloprasum* can induce high antifungal activity against one of the most harmful phytopathogens of economic importance and hence, could be used as potential biofungicide. Further investigation to isolate pure compounds from water extract having best antifungal activity could be conducted to formulate an effective and economical biofungicide.

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## AUTHORS' CONTRIBUTIONS

Dr. Sharma devised the conceptual idea which was further carried out in the laboratory by Er. S. Khan followed by developing draft Manuscript. Dr. Sharma supervised the technical details and finalized the manuscript. Er. A. Kaur procured the plant material and maintained the stock simultaneously prepared the extracts.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest in the publication.

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