ANTIFUNGAL POTENTIAL OF ETHANOL EXTRACTS OF ALLIUM SATIVUM AND ALLIUM AMPELOPRAansom

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ABSTRACT

Objective: In vitro analysis of Allium sativum and Allium ampeloprasum was performed to evaluate their antifungal potential against Alternaria triticina (ITCC 5496), causative agent of leaf blight in wheat and Magnaporthe oryzae (ITCC 6808), causative agent of blast disease in rice.

Methods: Ethanol extracts of A. ampeloprasum and A. sativum were prepared by crushing their bulb in liquid nitrogen and then immersing them in 90% ethanol and 100% ethanol separately. The antifungal activity test was determined by quantitative assay using 96-well microtiter plate, and results were statistically analyzed using GraphPad Prism v. 5.03.

Results: A. triticina and M. oryzae showed above 90% and 95% growth inhibition, respectively, against the ethanol extracts of A. ampeloprasum. Conversely, growth inhibition of either fungus remained mostly below 35% against ethanol extracts of A. sativum at all tested concentrations.

Conclusion: Ethanol extracts of A. ampeloprasum have relatively higher antifungal potential than ethanol extracts of A. sativum and could be considered as a natural alternative to chemical fungicides.

Keywords: Ethanol extract, antifungal, Allium sativum, Allium ampeloprasum, Alternaria triticina, Magnaporthe oryzae.

INTRODUCTION

Allium sativum (garlic) is known for its antimicrobial properties including antibacterial, antifungal, antiprotozoal, and insecticidal properties [1]. Crude extracts of A. sativum were tested against the laboratory isolate of Candida albicans [2] and are referred as broad-spectrum antimycotic agents [3]. Allicin, an active component of garlic, found in water and ethanol extracts of A. sativum [4], is often reported for its effective antifungal properties in many research articles, such as, against Candida, Cryptococcus, Trichophyton, Microsporum, and Epidermophyton [5]. Ajoene, another compound derived from ethanol extracts of A. sativum is also reported as an excellent antifungal agent, when tested against Aspergillus niger, C. albicans and Paracoccidioides brasiliensis [6]. Despite its remarkable antifungal potential, A. sativum is neither preferred in agricultural field for protecting crops from pathogens nor as a food preservative; because, of its strong odor [7].

Conversely, Allium ampeloprasum (elephant garlic) has a much milder flavor and is less pungent than A. sativum [8]. It is known to be more beneficial than other Allium species [9]. Essential oil of A. ampeloprasum showed inhibitory effect on Rhodotorula sp. and Saccharomyces cerevisiae [10]. Cinnamic acid derivatives of A. ampeloprasum were reported to possess antifungal potency against A. niger, Penicillium italicum, Botrytis cinerea, and Trichoderma harzianum [11].

The fungal strains used in this study are Alternaria triticina and Magnaporthe oryzae, fungal phytopathogens of wheat and rice, respectively. A. triticina (phylum Ascomycetes) causes leaf blight disease which results in high yield losses and severe infection in wheat and barley [12]. While investigating the most prevalent foliar blight pathogens in India, A. triticina was reported as the second most frequent phytopathogen [13]. On the other hand, M. oryzae (phylum Ascomycetes) is rated at first place among the list of top 10 most harmful fungal phytopathogens [14]. It causes rice blast disease which is a predominant biotic stress, affecting the rice production worldwide [15]. Although the chief host of this fungus is rice, it is also reported to infect wheat causing wheat blast disease [16].

Ethanol extracts of numerous medicinal plants are often reported for their antifungal properties in many researches [17-19]. Ethanol extracts of A. sativum are known to show a significant inhibitory effect against A. niger, Aspergillus usus, C. albicans, Fusarium oxysporum, Metschnikowia fructicola, and Penicillium species [7,20,21]. Ethanol extracts of A. ampeloprasum are not yet reported for their antifungal properties. This study was primarily undertaken to evaluate the antifungal properties of ethanol extracts of A. ampeloprasum. Further, the ethanol extracts of A. ampeloprasum were studied in contrast to the ethanol extracts of A. sativum, against two extremely harmful cereal crop pathogens, viz., A. triticina and M. oryzae, to study their potential as antifungal agents and to develop an efficient antifungal formulation.

MATERIALS AND METHODS

Biological material

A. sativum (garlic) and A. ampeloprasum (elephant garlic) garlic species were obtained from Dosanjh Agricultural Research and Development Farm, Punjab. Pure cultures of A. triticina (ITCC 5496) and M. oryzae (ITCC 6808) were used in this study.

Maintenance of fungal culture and spore isolation

Pure cultures were refreshed and maintained on potato dextrose agar slants and plates on regular basis. The cultures were streaked on sterile potato dextrose agar plates and kept in incubator at 27°C for 5-10 days depending on the growth rate of each fungus, once grown they were stored at 4°C. Fungal cultures were refreshed twice a month to avoid contamination. Spores were isolated from the cultures grown on potato dextrose agar plates and stored at 4°C in a sterile test tube [22].
Preparation of ethanol extracts

Bulb of both the garlic species was first washed with tap water followed by distilled water thoroughly. They were grounded to fine powder in liquid nitrogen using mortar and pestle and stored carefully at −20°C. 25 g of powdered material from each species was soaked in 50 ml of 90% ethanol and 100% ethanol separately and was kept at room temperature for 24 hrs; further, they were filtered using Whatman No. 1 filter paper. The filtrate was heated at 35-45°C using water bath, to completely remove any residues of ethanol and obtain dried powder. It was then weighed and dissolved in equal amount of distilled water to obtain 1 g/ml concentration of ethanol extracts. These extracts were stored in the form of aliquots at 4°C for further testing.

Antifungal activity

The antifungal activity test was performed by quantitative-assay [22] with little modifications. Growth inhibition was measured in sterile 96-well microtiter plate arranged in eight series A to H, each one with 12 wells numbered 1 through 12. Potato dextrose broth was used as medium to support the growth of fungal spores and also as a medium to make extract and spore suspension. Fungal spores were suspended in PDB and spore count was optimized to 2×10⁶ spores/ml, likewise, ethanol extract was also brought up to a concentration of 200 µg/ml of PDB from an initial concentration of 1 g/ml of water. 200 µl of extract suspension was added in all wells of series A, rest of the plate was filled with 100 µl of PDB. Now 100 µl of extract suspension from series A was added to series B and so on till series G to make dilutions in subsequent wells, remaining 100 µl extract suspension was discarded from series G. Add 100 µl of spore suspension in the first three wells of all the series and 100 µl of PDB in next three wells of all the series to provide dilutions of ethanol extract ranging from 100 µg/ml to 1.5625 µg/ml in series A to G, respectively, leaving series H as control. The first three wells of each series from A to G was referred as test and the next three wells as test blank; similarly first three wells of series H were referred as control and the next three wells as control blank. Remaining six wells of each series could be used to test another ethanol extract while following the pattern mentioned earlier. All tests were carried out in triplicates. Titer plates were covered with lid, sealed using parafilm, and kept at 27°C in B.O.D. incubator for 48 hrs after which readings were taken at 595 nm using Microplate reader (Biorad).

Calculation for percentage growth inhibition, minimal inhibitory concentration (MIC₅₀) and MIC₉₀

Percentage growth inhibition was determined based on the equation \[(\text{MIC} - \Delta T)/\Delta C\] × 100 where, ΔC is the average absorbance of the control microculture minus the average absorbance of control blank and ΔT is the average absorbance of the test microculture minus the average absorbance of test blank [23]. Growth inhibition was presented in the form of (mean±standard deviation [SD]) %, where SD is the standard deviation. MIC₅₀ and MIC₉₀ values were determined using the graphing software GraphPad Prism v. 4.4.2 for the ethanol extracts that showed greater antifungal activity. Minimum concentration of ethanol extract showing 50% and 90% growth inhibition were considered as MIC₅₀ and MIC₉₀ values, respectively.

Statistical analysis

Two-way analysis of variance (ANOVA) was performed to measure the significant difference among the different concentrations of the ethanol extracts against percentage growth inhibition, due to the effect of the ethanol extracts on each fungus. The data were gathered and were statistically analyzed using statistical software GraphPad Prism v. 5.03 at 95% confidence interval.

RESULT AND DISCUSSION

Test results showed that the maximum growth inhibition of *A. triticina* occurred by 100% ethanol extract of *A. ampeloprasum*, while other extracts showed less than 40% growth inhibition at all tested concentration. Lowest growth inhibition of (0.6±0.3) % was shown by 100% ethanol extract of *A. sativum* at 3.125 µg/ml concentration, while it showed growth inhibition of (21.3±0.4) % at a concentration of 12.5 µg/ml. 90% ethanol extract of *A. sativum* also showed low antifungal activity, with a minimum growth inhibition of (9.2±0.3) % at a concentration of 12.5 µg/ml and maximum growth inhibition of (12.5±0.3) % at 1.5625 µg/ml concentration. Similarly, 90% ethanol extract of *A. ampeloprasum* also showed low antifungal activity against *A. triticina* showing 15-32% growth inhibition at the test concentrations. Contrariwise, 100% ethanol extract of *A. ampeloprasum* showed highest growth inhibition of (91.6±1.3) % at a concentration of 1.5625 µg/ml. The lowest growth inhibition showed 100% ethanol extract of *A. ampeloprasum* was (44.9±0.4) % at 6.25 µg/ml concentration, which is much higher than the maximum growth inhibition shown by other extracts against *A. triticina* (Fig. 1), details are provided in Table 1.

The ethanol extracts of *A. sativum* showed relatively lower antifungal activity, though 100% ethanol extract of *A. sativum* managed to show 50% growth inhibition at 99.17 µg/ml concentration of *M. oryzae*. 90% ethanol extract of *A. sativum* promoted the growth of *M. oryzae* at all the test concentrations, except at 3.125 µg/ml concentration, where it showed (20.8±4.0) % and at 1.5625 µg/ml concentration, where (23.35±2.8) % of growth inhibition was attained. Ethanol extracts of *A. ampeloprasum*, both 90% and 100%, showed significantly higher antifungal activity against *M. oryzae*. The highest antifungal activity by 90% ethanol extract of *A. ampeloprasum* was observed at 100 µg/ml concentration showing (95.5±0.1) % growth inhibition of *M. oryzae*, while the highest inhibition shown by 100% ethanol extract of *A. ampeloprasum* was at 12.5 µg/ml concentration showing (91.3±0.3) % growth inhibition of *M. oryzae* (Fig. 2), details are provided in Table 2.

MIC₅₀ and MIC₉₀ values for 100% ethanol extract of *A. ampeloprasum* against *A. triticina* were observed at 1.706 µg/ml and 5.708 µg/ml.
respectively (Fig. 3). MIC\(_{50}\) and MIC\(_{90}\) values were also determined for 90% and 100% ethanol extract of \(A.\) ampe\(l\)oprasum against \(M.\) oryzae. MIC\(_{50}\) was observed at 17.333 \(\mu\)g/ml for 90% ethanol extract of \(A.\) ampe\(l\)oprasum, and MIC\(_{90}\) was observed at 75.116 \(\mu\)g/ml. Similarly, MIC\(_{50}\) for 100% ethanol extract of \(A.\) ampe\(l\)oprasum was observed at 7.297 \(\mu\)g/ml, and MIC\(_{90}\) was observed at 12.336 \(\mu\)g/ml (Table 3).

The existence of antifungal activity in the extracts may be attributed to the presence of allin in which is known for its broad spectrum antifungal activity. It is a sulfur-containing compound which is responsible for the pungent smell of damaged or crushed garlic [24]. Allin is the major compound, naturally present in garlic which is broken down into allin, ammonium, and pyruvate under the action of alliinase enzyme [25]. Allin is known to inhibit the germination of fungal spores as well as the hyphal growth [5]. Ajoene, another major bioactive compound of garlic might also be attributed to the antifungal property of the extracts [6,26]. Ajoene is known to possess much stronger antifungal activity as compared to allin; however, it is not capable of inducing antibacterial effect [27]. Many another bioactive compound capable of inhibiting fungal growth might also be attributed to the antifungal activity of the extracts [26,28-30]. While, some extracts having poor or no inhibitory activity promoted the growth of the fungi, as seen in the case of Ipomoea extracts against Colletotrichum species [31].

**Fig. 3:** Minimal inhibitory concentration\(_{50}\) and MIC\(_{90}\) values of ethanol extracts of \(A.\) ampe\(l\)oprasum and \(A.\) sativum at different concentrations against Alternaria triticina and Magnaporthe oryzae shown in log-lin scale (y-axis is logarithmic to base 2). Error Bar indicates a standard error of ±5.0%.

**Table 1:** Antifungal activity of ethanol extracts of \(A.\) ampe\(l\)oprasum and \(A.\) sativum at different concentrations against \(A.\) triticina. Growth inhibition is shown in the form of (Mean±SD), where SD is standard deviation.

| Concentration (in \(\mu\)g/ml) | 90% Ethanol \(A.\) ampe\(l\)oprasum | 90% Ethanol \(A.\) sativum | 100% Ethanol \(A.\) ampe\(l\)oprasum | 100% Ethanol \(A.\) sativum |
|-----------------------------|---------------------------------|---------------------|-------------------------------|---------------------|
| 100                         | 15.20±0.4                        | 12.17±0.2           | 72.82±1.2                     | 19.86±0.6            |
| 50                          | 16.46±0.5                        | 10.58±1.4           | 74.41±0.4                     | 20.51±1.2            |
| 25                          | 27.13±1.7                        | 9.46±0.9            | 81.21±0.7                     | 20.42±3.9            |
| 12.5                        | 31.24±0.7                        | 9.28±0.3            | 55.48±0.7                     | 21.31±0.4            |
| 6.25                        | 22.47±0.9                        | 11.98±0.7           | 44.94±0.4                     | 9.98±0.3             |
| 3.125                       | 28.53±1.4                        | 9.65±0.7            | 74.08±1.2                     | 0.65±0.3             |
| 1.5625                      | 31.75±0.3                        | 12.49±0.3           | 91.61±1.3                     | 11.61±0.4            |

**Table 2:** Antifungal activity of ethanol extracts of \(A.\) ampe\(l\)oprasum and \(A.\) sativum at different concentrations against \(M.\) oryzae. Growth inhibition shown in the form of (Mean±SD), where SD is standard deviation.

| Concentration (in \(\mu\)g/ml) | 90% Ethanol \(A.\) ampe\(l\)oprasum | 90% Ethanol \(A.\) sativum | 100% Ethanol \(A.\) ampe\(l\)oprasum | 100% Ethanol \(A.\) sativum |
|-----------------------------|---------------------------------|---------------------|-------------------------------|---------------------|
| 100                         | 95.57±0.1                       | -52.17±0.9          | 12.15±1.2                     | 50.27±0.6            |
| 50                          | 84.37±0.9                       | -45.57±1.3          | 25.26±3.6                     | 33.90±0.6            |
| 25                          | 73.45±2.4                       | -33.44±4.3          | 79.76±1.5                     | 30.82±1.3            |
| 12.5                        | 35.22±4.0                       | -23.97±1.8          | 91.30±0.3                     | 14.20±1.2            |
| 6.25                        | 20.50±1.9                       | -14.00±2.0          | 41.69±1.6                     | -54.86±1.3           |
| 3.125                       | 11.16±0.9                       | 20.83±4.0           | 33.05±2.4                     | -74.44±0.6           |
| 1.5625                      | -3.95±1.6                       | 23.35±2.8           | -33.78±2.4                    | -85.32±1.6           |
The two-way ANOVA confirmed that means of all the comparisons are significantly different, p<0.0001 was obtained, in the case of both A. triticina and M. oryzae. Based on which, it is determined that each ethanol extract shows a different level of antifungal activity at different concentration against A. triticina as well as against M. oryzae.

Table 3: MIC<sub>90</sub> and MIC<sub>90</sub> values of ethanol extracts of A. ampeloprasum and A. sativum at different concentrations against A. triticina and M. oryzae

| Name of fungi | MIC 90% Ethanol A. ampeloprasum | 90% Ethanol A. sativum | 100% Ethanol A. ampeloprasum | 100% Ethanol A. sativum |
|---------------|---------------------------------|------------------------|-----------------------------|------------------------|
| A. triticina   | 90                              | nd*                    | 1.706*                      | nd                     |
| M. oryzae     | 50                              | nd                     | 5.708                       | nd                     |
|               | 90                              | 75.116                 | 12.336                      | 99.169                 |
|               | 50                              | 17.333                 | 7.297                       | nd                     |

*nd means not determined, concentration of ethanol extracts is in µg/ml

CONCLUSION

The ethanol extracts of A. ampeloprasum showed substantial amount of antifungal activity against both fungi, sufficient enough to yield MIC<sub>90</sub> and MIC<sub>90</sub> values. A. triticina showed insignificant amount of inhibition in its growth when treated with ethanol extracts of A. sativum, in addition, M. oryzae showed growth, instead of inhibition, when treated with ethanol extracts of A. sativum. Therefore, it could be concluded that the ethanol extracts of A. ampeloprasum showed better antifungal activity against A. triticina as well as against M. oryzae as compared to A. sativum. Hence, they could be used as potential fungicide, not only because of their superior antifungal property but also because of their less pungent and harmless nature against humans which is desirable of a fungicide when being used in a crop field. Further, study on the identification and extraction of pure bioactive compounds from the ethanol extracts, as well as use of other solvents for the extraction of ethanol extracts from A. ampeloprasum could also be implemented to study the antifungal properties of A. ampeloprasum extensively.

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