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Research Article

ANTIFUNGAL POTENTIAL OF ETHANOL EXTRACTS OF *ALLIUM SATIVUM* AND *ALLIUM AMPELOPRASUM*

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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*.

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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 ustus, 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 ground 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 $2*10^6$ 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 (MIC_{50}) and MIC_{90}

Percentage growth inhibition was determined based on the equation ([ΔC-ΔT]/ΔC)* 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. MIC_{50} and MIC_{90} values were determined using the graphing software Graph 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 MIC_{50} and MIC_{90} 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, although 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.

MIC_{50} and MIC_{90} values for 100% ethanol extract of *A. ampeloprasum* against *A. triticina* were observed at 1.706 µg/ml and 5.708 µg/ml,

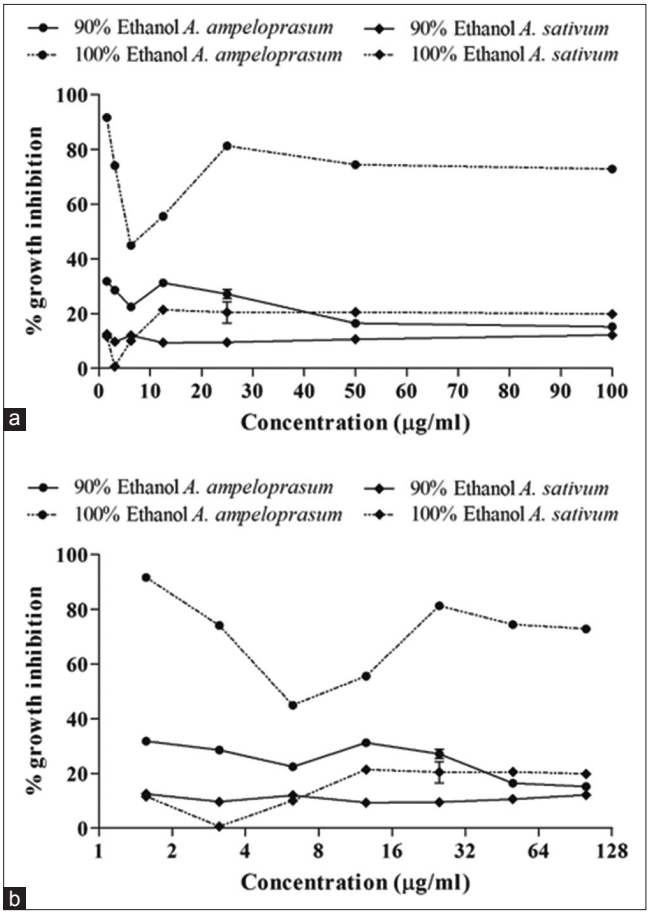

Fig. 1: Effect of the ethanol extracts on the growth of *Alternaria triticina* (a) lin-lin scale (b) lin-log scale (x-axis logarithmic to the base 2)

respectively (Fig. 3). MIC_{50} and MIC_{90} values were also determined for 90% and 100% ethanol extract of *A. ampeloprasum* against *M. oryzae*. MIC_{50} was observed at 17.333 µg/ml for 90% ethanol extract of *A. ampeloprasum*, and MIC_{90} was observed at 75.116 µg/ml. Similarly, MIC_{50} for 100% ethanol extract of *A. ampeloprasum* was observed at 7.297 µg/ml, and MIC_{90} was observed at 12.336 µg/ml (Table 3).

The existence of antifungal activity in the extracts may be attributed to the presence of allicin 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]. Alliin is the major compound, naturally present in garlic which is broken down into allicin, ammonium, and pyruvate under the action of alliinase enzyme [25]. Allicin 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 allicin; 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].

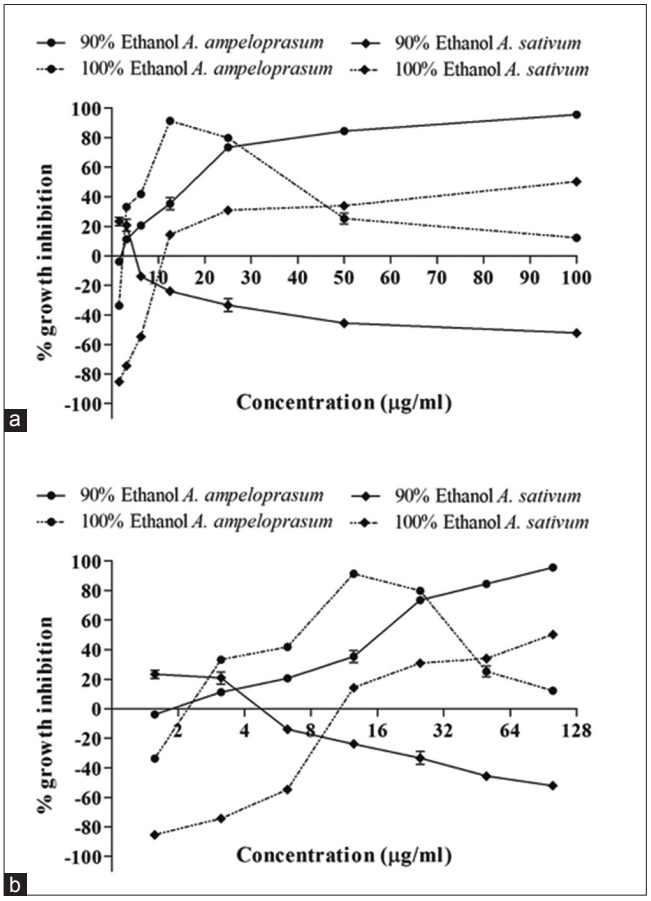

Fig. 2: Effect of ethanol extracts on the growth of *Magnaporthe oryzae*. (a) lin-lin scale (b) lin-log scale (x-axis logarithmic to the base 2)

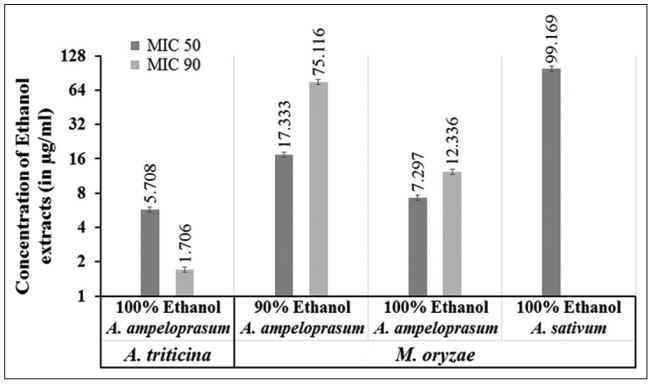

Fig. 3: Minimal inhibitory concentration$_{90}$ and MIC_{50} values of ethanol extracts of *Allium ampeloprasum* and *Allium sativum* at different concentrations against *Alternaria triticina* and *Magnaporthe oryzae* shown in log-lin scale (y-axis is logarithmic to base 2). Error Bar indicates a standard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

Table 1: Antifungal activity of ethanol extracts of *A. ampeloprasum* 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 µg/ml)	90% Ethanol *A. ampeloprasum*	90% Ethanol *A. sativum*	100% Ethanol *A. ampeloprasum*	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. ampeloprasum* 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 µg/ml)	90% Ethanol *A. ampeloprasum*	90% Ethanol *A. sativum*	100% Ethanol *A. ampeloprasum*	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	79.70±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

Table 3: MIC_{90} and MIC_{50} 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*	nd	1.706*	nd
	50	nd	nd	5.708	nd
M. oryzae	90	75.116	nd	12.336	99.169
	50	17.333	nd	7.297	nd

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

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*.

CONCLUSION

The ethanol extracts of *A. ampeloprasum* showed substantial amount of antifungal activity against both fungi, sufficient enough to yield MIC_{50} and MIC_{90} 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 the ethanol extracts of *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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