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Antifungal Activity of Various Medicinal Plants against Late Blight of Potato from Ethiopia

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Authors' contributions

This work was carried out in mutual collaboration between both authors. Author MAA carried out all experimental work, data acquisition and analysis, literature searches. Author NMC was responsible for study concept, designing and coordinating the research and supervising the work. He was responsible for writing the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Emerging losses caused by late blight of potatoes need new approaches to control *Phytophthora infestans*. Searching for novel molecules with antifungal activity, twelve medicinal plants from Ethiopia were investigated against this pathogen.
Methodology: To accomplish this task, growth inhibitory effect of aqueous, ethanolous, and methanolous extracts of various medicinal plants were tested *in vitro* by applying agar well diffusion technique.
Results: All plant extracts except *Eucalyptus globules* and *Hagenia abyssinica* showed promising antifungal activity against the *P. infestans* mycelial growth. *Datura stramonium*, *Rhamnus prinoides* and *Moringa stenopetala* exhibited highest activity against *P. infestans*. Direct germination of zoosporangia was also significantly inhibited by all extracts of medicinal plants except *E. globules* and *H. abyssinica*. Methanolous extract of *Nicotinia tabacum* did not inhibited either mycelium growth or sporulation of the fungus.
Conclusions: Various products of proposed medicinal plants deserved to be reliable sources as antifungal agents and might play significant role for future practical applications in a socially and

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ecologically healthy management of late blight of potatoes. Further work in this direction can give insight into novel antifungal targets and molecules for prevention of *P. infestans* infection, especially against late blight of potato and tomato.

Keywords: Phytophthora infestans; antifungal activity; potato; sporocidal activity; medicinal plants.

1. INTRODUCTION

Ethiopia has good climatic and soil conditions that favor higher potato production. Potato has high potential for it significantly contributed to the reduction of food insecurity and improved nutrition in developing country like Ethiopia [1]. In Ethiopia, potato is cultivated as major crops around all parts of the country. Most especially the southern part of this country is the major contributor of fresh potato tubers to the capital city [2]. Despite of suitable natural conditions for potatoes farming in this vicinity, recently there has been increase in the loss of yield of potatoes due to several factors. One factor that is responsible for this loss is the infection to potato crops by a common disease i.e. late blight of potatoes caused by *Phytophthora infestans* [3]. The relative short gap between infection and lesion appearance causes frequent death of premature leaves and tuber disease. As a result, high potato yield losses have been reported [4].

To prevent yield losses in such important crop, it needs effective control measures like cultural measures, resistant varieties, and well-defined fungicidal sprays [5]. However, new challanges aroused due to development of acquired tolerance to phenilamide derivatives such as metalaxyl in populations of oomycetacuous plant pathogens [6]. Furthermore, management practices through fungicides application have brought environmental and economic side effects to the premising area where they are applied for control [7]. Such environmental risks cannot be applied directly to control pathogenesis, so that, they cause long term effects on the ecosystem [6]. In Africa 40-70% yield losses have been estimated due to late blight [7]. For more effective management of late blight, farmers have gradually adopted fungicide application, however, because of lack of knowledge on disease development and fungicides application, management practices are unsuccessful in developing countries [8].

Emergence of resistant strains of *P. infestans* and environmental and economic side effects of chemical fungicides are well known problems in pest management [3]. Infections caused by the fungal pathogen, *P. infestans*, are difficult to treat because of the same reasons [7]. Therefore, search for novel antifungal agents need to be continued to avoid such problems. Plants are reported as rich sources of bioactive compounds and are being explored for novel antimicrobial properties [9]. Use of novel plant substances against *P. infestans* may be an effective strategy. Various *in vitro* experiments have shown that extracts of medicinal plants possess potential inhibitory activities against fungal pathogens. Extracts of 26 plants were found to interfere with mycelium growth effectively of *P. infestans in vitro* [10]. Methylene chloride/methanol extracts of seven plants have shown *in vitro* and *in vivo* antifungal properties against tomato blight caused by *P. infestans* [11]. In another study by Messgo et al. [12] highlighted the *in vitro* and *in vivo* activity of aqueous extracts against late blight of potatoes. *In vitro* and greenhouse study was also evaluated against *P. infestans* [13]. To reduce this risk of chemical fungicide, use of natural substances derived from plants would primarily be assumed to be reliable, eco-friendly, economically benefit for farmers and effective alternative methods to prevent and control potato late blight. This study was performed to overcome such problems by using selected local medicinal plants. Moreover, no systematic efforts are made to explore the effect of various medicinal plants on fungal infection of *P. infestans* in Ethiopia. In this study, for the first time we report the effect of various medicinal plants on mycelium growth and spores of *P. infestans*.

2. MATERIALS AND METHODS

2.1 Plant Materials and Extract Preparation

All plant materials used in this study were collected from Dilla town (6°24'43" N, 38°18'02" E) of southern region of Ethiopia in August 2014. Twelve different plant material including leaves, seeds, and fruits parts of different medicinal plants were used for the study and are highlighted in Table 1. The plants were confirmed at genus and species level by botanist and were utilized for the studies. After harvesting, the plant

materials were cleaned with tap water to clear it of fragments of soil, then it was left to dry away from direct sunlight, at ambient temperature and in open air for three weeks. The dried plants were grind to a fine powder in a mixer grinder (Bajaj Rex 500, India), and stored at room temperature in closed 2000 ml glass jars in the dark, at 20℃ until used. Fifty grams of the powdered, dried plant samples were weighed and placed into 1000 ml Erlenmeyers flasks and then 500 ml of methanol, ethanol and distilled water (Hi-Media Ltd. Mumbai) was added to the flask. The flaks were closed with a cotton balls and covered with aluminum foil and then placed on a Soxhlet extractor (Micro Technologies, Ambala, India) for extraction for 18 hours and then the suspension was filtered through two layers of cheese cloth into different 250 ml of evaporating flasks. Excess of methanol and ethanol were evaporated using a rotary evaporator (ScienceTech Instruments, New Delhi, India) at 32 ± 2℃, whereas aqueous plant filtrates were concentrated to powder using a freeze drier (Optics Technology, New Delhi, India). After evaporation the stock was prepared by dissolving 100 mg of dry extracts in 1 ml of appropriate solvent. These stock suspensions were stored at 4℃ and used within four days.

2.2 Collection of Fungal Pathogen

The infected leaves and tubers of potato were collected from various potato fields around Dilla region. 100 ml of Potato Dextrose Agar (PDA) (Himedia Ltd. Mumbai, India) was prepared, and was poured into Petri dishes and after solidifying the media, infected potato tuber and leaves were inoculated aseptically and incubated for 24 to 48 hrs at 37℃. After incubation, growth of *P. infestans* was confirmed on the basis of sporulation property as well as biochemical properties by referring standard protocols [14]. The culture was stored at 4℃ in refrigerator and was utilized for the further experiments.

2.3 Agar Well Diffusion Assay

The effect of the extracts on mycelial growth was determined by using agar well diffusion technique. Individual ingredients of PDA were added to 1000 µl of distilled water and autoclaved at 121℃ for 21 minutes, subsequently poured in sterile Petri plates. Once the media get solidify, the plates were incorporated with 4 wells with the help of sterile borer (5 mm diameter), then a loopful suspension of *P. infestans* was spread uniformly with sterile cotton swab. Following this, the wells were poured with different concentrations of various extracts i.e. 1, 2, 4, and 8 mg/ml and leave in refrigerator for 5 minutes to diffuse bioactive materials. Wells without any extract served as control. Finally, the plates were incubated at 30℃ for 48 hrs, then the inhibition zone was measured [15].

2.4 Sporocidal Activity

To study the effect of plant extracts on spore germination of *P. infestans*, cavity slide method was used [14]. Briefly, spore suspension was prepared by adding distilled water directly into the Petri dishes of 14 days old cultures of *P. infestans.* One drop (0.1 ml) of spore suspension and one drop (0.1 ml) of different concentrations of plant extracts was taken on separate cavity slides. Sterilized distilled water was used as control. The slides were incubated at 30℃ in moist chambers (in large Petri dishes containing blotting papers blotted with sterile water) for 24 hrs. After incubation germinated sporangia was counted under light microscope at 40 x magnifications and percentage inhibition of spore

Table 1. Medicinal plants used for the experiment analysis against *P. infestans*

Scientific name	Vernacular name	Local name	Family	Plant parts
Capsicum annum	Hot peppers	Miximixo	Solanaceae	Fruits
Citrus limon	Lemon	Lomae	Rutaceae	Fruits
Cymbopogon citratus	Lemon grass	Xinoqiimama	Poaceae	Seeds
Datura stramonium	Jimson weed	Atsefareceae	Solanaceae	Leaves
Eucalyptus globules	Blue gums	Barzafae	Myrtaceae	Leaves
Hagenia abyssinica	Hagenia	Kossae	Rosaceae	Leaves
Lepidium sativum	Garden cress	Faxxoo	Brassicacee	Seeds
Moringa stenopetala	Cabbage-tree	Shifferaw	Moringacea	Leaves
Nicotiana tabacum	Tobacco	Tambo	Solanaceae	Leaves
Ocimum lamiifolium	Ocimum	Damakase	Lamiaceae	Leaves
Rhamnus prinoides	Dog wood	Geshae	Rhamnaceae	Leaves
Ruta chalepensis	Fringed rue	Xenadamae	Rutaceae	Leaves

was calculated by comparing with that of control by using following formula:

Percentage Inhibition (%) = (Growth in control - Growth in treatment) / Growth in control x 100

2.5 Statistic Analysis

All the experiments were done in triplicate and standard deviation from the mean was calculated. Effect of plant extract on fungus was analyzed using Two-way Anova (Bonferroni posttests) by using SPSS software windows version 16.0. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Effects of different crude extracts on growth of *P. infestans* mycelium

Extracts of two of twelve medicinal plants i.e. *Eucalyptus globules* and *Hygenia abyssinica* did not produced any anti-mycelium activity against *P. infestans*. Whereas, ten medicinal plants significantly ($P = 0.0018$) inhibited the mycelium growth of *P. infestans* in concentration dependent manner. Aqueous extract of *Datura stramonium* showed strongest inhibition i.e.17 mm, followed by *Nicotiana tabacum, Cymbopogon citratus*, *Moringa stenopetala*, *Capsicum annum, Ocimum lamiifolium*, *Lepidium sativum*, and *Rhamnus prinoides* which showed 16, 13.5, 12, 11.5, 11.5, 11, and 10 mm zone of inhibition at 8 mg/ml of concentration as compared to the control. Among them extracts of *Nicotiana tabacum* and *Datura stramonium* were found to be effective at 4 mg/ml relating to the others as 11.5 and 10.5 mm zone of inhibition were recorded. Whereas, *Ruta chalepensis* and *Citrus limon* indicated moderate inhibition at 8 mg/ml and 4 mg/ml of extracts. All aqueous extracts were not much effective at 2 mg/ml and 1 mg/ml (Table 2). Results of ethanol extract on mycelium growth revealed highest inhibition by *R. prinoides* (15 mm), continued by *D. Stramonium, L. sativum, C. limon, R. chalepensis, C. annum, M. stenopetala, C. citrates, N. tabacum,* and *O. lamiifolium* were found to reduced the growth of *P. infestans* mycelium more effectively as compared to control. They gave 12.5, 12.5, 12.5, 12, 11, 10, 8, 7 and 6.5 mm values of zone of inhibition respectively at 8 mg/ml of concentration. The data also indicated that ethanol extracts of *R. prinoides, C. limon, D. stramonium, C. annum,* and *L. sativum* produced effective results at 4 mg/ml of concentration where, 14, 11.5, 10.5, 9, and 8.5 mm respectively zone of inhibition was seen. At 2 mg/ml *R. prinoides, C. limon, D. stramonium*, and *C. annum* also produce measurable i.e. 12, 10, 9, and 7 mm of inhibition, respectively as compared to the control. Whereas, at 1 mg/ml all extracts were resulted in moderate effect on growth of mycelium as compared to control (Table 2). Methanol extracts of *N. tabacum* failed to produce zone of inhibition at all concentrations. While extract of *D. stramonium* (15.5 mm) showed highest inhibition, followed by *O. lamiifolium*, *R. chalepensis*, *C. annum, C. limon, R. prinoides, M. stenopetala, L. sativum* and *C. citrates* where 14.5, 12.5, 11, 11, 11, 10.5, 7 and 6.5 mm values of zone of inhibition was reported, respectively. Also, *D. stramonium, O. lamiifolium, C. limon, R. prinoides* and *M. stenopetala* reduced growth of mycelium at 4 mg/ml and produces value of 12, 11, 9.5, 9.5, and 9 mm zone of inhibition respectively. At 2 and 1 mg/ml, extracts of *D. stramonium* and *O. lamiifolium* gave 10.5 and 9.5 mm of inhibition zone.

3.1.2 Effect of various plant extracts on spore germination of *P. infestans*

Effect of different medicinal plant extracts were studied in presence of various concentrations on spore germination. Among twelve (12) medicinal plants studied, ten medicinal plants produced effective results. All the extract studied produces sporocidal results in concentration dependent manner ($P < 0.05$). Extract of *Eucalyptus globules* and *Hygenia abyssinica* did not affect spores of *P. infestans* as 90% of spore germination was noted at higher concentration. Aqueous extracts of *D. stramonium, N. tabacum*, *C. annum*, *L. sativum*, *C. citrates, R. chalepensis*, *M. stenopetala*, *C. limon*, *O. lamiifolium*, *R. prinoides* significantly inhibited average spore formation i.e. 75-50% at 8 mg/ml of concentration. Addition of 4 mg/ml of concentration of these effective medicinal plants halted spore germination by 50-30% while 2 and 1 mg/ml stopped sporulation by 30-0% (Table 2). Ethanol extract of *R. prinoides*, *C. limon*, *L. sativum, R. chalepensis*, *M. stenopetala*, *N. tabacum*, *D. stramonium, C. annum*, *C. citrates* and *O. lamiifolium* significantly (more than 50%) halted spore germination at 8 mg/ml of concentration. While, 4 mg/ml, 2mg/ml and 1 mg/ml did not produced adverse effect which resulted in 40-0% of sporulation (Table 2). Methanol extract of *N. tabacum* failed to halt sporulation in *P. infestans*, while methanol

extracts of *M. stenopetala*, *L. sativum*, *C. citrates*, *C. limon*, *O. lamiifolium, R. prinoides, C. annum* and *D. stramonium* at 8 mg/ml of concentration produced more than 50% of inhibition of spores. While the above mention plant extract at lower concentrations halted spore germination by 40-5% in comparison to control (Table 3).

3.2 Discussion

Potato late blight caused by *P. infestans* is the major threat affection lot of potato production [16]. The development of disease resistance by *P. infestans* to conventional fungicide and environmental contamination problems creates pressure on farmers to adopt new control strategy to reduce fungal infection to support potato production [17]. Plants are able to produce various classical compounds. Besides the classic primary metabolites, they can synthesize and accumulate secondary metabolite, whose physiological function are not always obvious but represents a wide range of important molecules in agriculture within the framework of phyto-protection against various important pathogens.

In this study, ethanol extract of medicinal plant was found to possess good anti-mycelium activity at higher concentration (8 and 4 mg/ml). Whereas, at lower concentration (2 to 1 mg/ml) were found ineffective within an exception of *C. limon,* and *R. prinoides.* The current study showed that most of the ethanol extracts have antifungal activity against mycelium growth of *P. infestans.* The finding of this study is in agreement with the study of previously reported authors, who suggested that same plants may have different antimicrobial activity in different solvents [18]. For example ethanol extract of some medicinal plants such as *Aloe vera*, were found to be effective against some fungal pathogens in comparisons to other solvent extract [19]. The possible reason for this effect that each plants contains different components in the form of secondary metabolites that have different characteristic effect in various solvents which varies on the basis of physical and chemical properties. These properties have different modes of action on different microbes and may results in variable results [20]. The methanol extracts of *D. stramonium* and *O. lamiifolium* was the most effective extracts that reduced growth of *P. infestans* mycelium. From this *D. stramonium* has resulted in higher zone of inhibition. This result was similar with the work done by Sharma et al. (2013) [21] who aimed to evaluate the *in vivo* and *in vitro* antimicrobial properties of different plant parts (alcoholic extracts), whole plants (extracted sequentially with different organic solvents) of *D. stramonium.* For this, antimicrobial properties were tested against bacteria *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa* and the fungal strains such as *Aspergillus flavus*, *A. niger, Fusarium culmorum* and *Rhizopus stolonifer.* All the solvent extracts showed significant activity against all the tested fungus. Methanolic extract is most active against all fungus.

Sporulation plays an important role in the life cycle of *P. infestans* [5]. Screening of plant molecules for sporocidal property can be an alternative strategy to avoid the use of standard antifungals. Results of our study demonstrated that various plants posse's sporocidal property in concentration dependent manner suggesting that plants extract have good sporocidal properties. Our results are in the line of Rashid et al. [5] who reported that the extract of *Azadarictus indica* has good inhibitory effect against *P. infestans* spore germination in concentration dependant manner.

The presence of antimicrobial substances in the higher plants is well established [22]. Plants have provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health as well as in reducing the effects of the plant pathogens. Phytomedicine can be used for the treatment of various diseases as is done in case of Unani and Ayurvedic system of medicines or it can be the base for the development of a medicine, a natural blueprint for the development of a drug [23]. Successive isolation of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The traditional healers use primarily water as the solvent but we found in this study the plant extracts by methanol provided more consistent antimicrobial activity compared to those extracted by ethanol and water. This might have resulted from the lack of solubility of the active constituents in aqueous solutions while methanol extract showed some degree of antifungal activity. Further trials using solvents of various polarities will explore the effects of solvent composition on extract efficacy [24]. This plant can be further subjected to isolation of the therapeutic antimicrobials and carry out further pharmacological evaluation to resolve the problems of fungal pathogens.

Table 2. Effects of different medicinal plants growth of *P. infestans* mycelium

Medicinal plants	Zone of inhibition (mm)											
	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml
	Aqueous extract				Ethanol extract				Methanol extract			
NT	16(±1.4)	11.5(±1)	6(±0.4)	2.5(±1.7)	7(±1.1)	5.5(±0.7)	3(±1)	1.5(±0)	0(±0)	0(±0)	0(±0)	0 (±0)
RC	7.5(±2.8)	6.5(±1.4)	4.5(±2.1)	3.0(±2.1)	12(±0.7)	6(±0.7)	3.5(±0.5)	2.0(±0.6)	12.5(±1.8)	6.5(±0.8)	2(±0)	0(±0)
MS	12(±1.7)	6.5(±1.4)	3.5(±1.4)	1.0(±0.6)	10(±0.8)	5.5(±1.4)	4.5(±2)	3.5(±0.4)	10.5(±0.9)	9(±1.5)	3.5(±0.4)	1(±0)
DS	17(±0.6)	10.5(±0.7)	4.5(±1.5)	0(±0)	12.5(±1.2)	10.5(±2.1)	9(±0.6)	5(±0.5)	15.5(±0.2)	12(±2.1)	10.5(±1.2)	4.5(±0.7)
CA	11.5(±0.7)	7.5(±0.7)	4.5(±0.7)	0(±0)	11(±0.4)	9(±0.6)	9(±0.7)	4.5(±0.1)	11(±2.1)	4.5(±0.6)	2(±0.4)	0(±0)
LS	11(±2)	7(±0.6)	5(±0.7)	0(±0)	12.5(±1.7)	8.5(±0.8)	3.5(±0.6)	2(±0)	7(±0.5)	2(±0)	0(±0)	0(±0)
CC	13.5(±1.6)	6.5(±0.4)	4(±0.6)	0(±0)	8(±0.5)	3.5(± 0)	2(±0.4)	0(±0)	6.5(±0.6)	3(±0.3)	1(±0)	0(±0)
CL	8.5(±2.0)	5.5(±1.1)	2(±0)	1.0(±0.1)	12.5(±1.6)	11.5(±0.5)	10(±0.8)	5(±0.7)	11(±0.5)	9.5(±0.7)	4.5(±0.7)	2(±0)
OL	11.5(±0.6)	7.5(±2.5)	0(±0)	0(±0)	6.5(±0.3)	4.5(± 0.2)	2.5(±0.2)	0(±0)	14.5(±0.5)	11(±0.9)	9.5(±1.4)	3.5(±0.4)
RP	10(±0.7)	8.0(±1.0)	0(±0)	0(±0)	15(±0.2)	14(±1.6)	12(±1.5)	6.5(±1.0)	11(±1.7)	9.5(±1.5)	5.0(±0.5)	0(±0)
EG	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)
HA	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)	0(±0)

**Values indicate are the mean of triplicate. Values indicated in parenthesis represent standard deviation. Agar plates were incubated at 30 ℃ for 48 hour s and after incubation zone of inhibition was easured in mm. NT: Nicotiana tabacum; RC: Ruta chalepensis; MS: Moringa stenopetala; DS: Datura stramonium; CA: Capsicum annum; LS: Lepidium sativum; CC: Cymbopogon citratus; CL: Citrus limon; OL: Ocimum lamiifolium; RP: Rhamnus prinoides; EG: Eucalyptus globules; HA: Hygenia abyssinica*

Table 3. Sporocidal property of various medicinal plants against *P. infestans*

Medicinal plants	Percentage inhibition of spores											
	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml
	Aqueous extract				Ethanol extract				Methanol extract			
NT	68	48	6	11	57	25	10	0	3	2	5	2
RC	54	28	5	0	49	30	20	13	58	32	21	14
MS	66	32	3	0	67	25	18	13	84	25	23	9
DS	90	41	26	0	64	41	30	18	87	39	17	5
CA	53	42	22	0	54	25	15	12	52	28	25	12
LS	60	47	25	0	54	42	37	18	60	18	12	7
CC	57	45	20	0	66	38	19	10	67	25	14	9
CL	55	41	14	0	63	44	33	26	54	37	24	11
OL	49	42	20	0	52	24	17	10	49	31	27	16
RP	54	31	11	0	77	33	34	22	72	22	14	6
EG	7	3	0	0	5	3	0	0	9	0	0	0
HA	1	1	0	0	1	2	0	0	4	1	0	0

Values indicate are the mean of triplicate. Concentrations of different medicinal plant extract were tested against spore germination of P. infestans. Slides were incubated at 30 ℃ for 24 hours and after incubation spore were observed under microscope and percentage inhibition was calculated by comparing with that of control (without extract). NT: Nicotiana tabacum; RC: Ruta chalepensis; MS: Moringa stenopetala; DS: Datura stramonium; CA: Capsicum annum; LS: Lepidium sativum; CC: Cymbopogon citratus; CL: Citrus limon; OL: Ocimum lamiifolium; RP: Rhamnus prinoides; EG: Eucalyptus globules; HA: Hygenia abyssinica

4. CONCLUSION

In the end, all plant extracts except *E. globules* and *H. abyssinica* exhibited promising antifungal activity against the *P. infestans* mycelial growth. Direct germination of zoosporangia was also significantly inhibited by all extracts of medicinal plants apart from *E. globules* and *H. abyssinica*. Various products of proposed medicinal plants deserved to be reliable source for active antifungal agents and might play significant role for future practical applications in a socially and ecologically healthy management of late blight of potatoes. Conclusively, our study analyzed the activity of various medicinal plants against *P. infestans*. Its sporocidal property and potential to inhibit *P. infestans* mycelium is revealed for the first time from Ethiopia. Further work in this direction may give insight into novel antifungal targets and molecules for prevention of *P. infestans* infection, especially against late blight of potato and tomato.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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