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In Vitro Antifungal Activity of Neem Products Against *Phytophthora Infestans*

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Abstract: Four neem (*Azadircht a indica* A. Juss) products namely crude *neem* seed oil, nimbokil, crude neem seed oil terpenoid extract and neem leaf decoction were tested for their *in vitro* activity against mycelial growth, sporangial germination and sporangial production of *Phytophthora infestans.* All the products were found highly effective against the different life stages of the fungus. The compounds were evaluated both with and without being heat sterilized along the medium. The heat sterilized compounds were comparatively less effective as compared to the ones which were used without heat sterilization. The results show that neem products have potential for the management of potato late blight disease.

Key words: *Phytophthora infestans,* Late blight, *Azadirchta indica,* Neem

Introduction

Conventional management of late blight and importance of neem: Conventionally control of late blight disease has mostly been tried by using synthetic fungicides. Repeated use of chemicals led to ecological hazards in addition to development of fungicide resistance in fungi (Ghewande, 1995). Economic and environment friendly approach for the management of potato lateblight is needed to be explored. The use of different botanicals including neem *(Azadirachta indica* A. Juss) based preparations is inovative approach for the management of potato late blight. Various neem products based on the extracts of kernels, leaves, bark, flowers and wood are being utilized for the control of different pest pathogens. The biological activity of these products is mainly due to the presence of isoprenoids. There are hundreds and thousands of chemicals in this group which are not only effective against different pests and pathogens but are also unlikely to leave any harmful effect on the environment (Kazmi *et al*., 1991). Some of these chemicals include azadirachtin, meliantriol, salannin of laminoid group which have antifungal, antibacterial, antiviral, antifeedant, repellant and insecticidal properties (Roy, 1995). These products are proved to have significant role in controlling diseases and minimizing the environmental hazards (Eswaramurthy *et al*., 1989). Mariappan *et al*. (1988) reported the antimycotic activities of different neem based products. Keeping in view the environment friendly nature, fungicidal properties and cheap availability of neem, its products were evaluated for their effectiveness against different growth stages of potao late blight pathogen *Phytophthora infestans.*

Materials and Methods

Four neem products namely crude neem seed oil, nimbokil (a commercial formulation of neem oil), crude neem seed oil terpenoid extract and neem leaf decoction, were evaluated for their fungicidal properties against different life stages of *P. infestans* under in-vitro conditions.

Neem seed oil and nimbokil were obtained from Grain Storage Laboratory, Tropical Agricultural Research Institute, Karachi, Pakistan, while crude neem seed oil terpenoid extract was obtained from Department of Biochemistry and Molecular Biology, University of Glasgow, U.K. For neem leaf decoction leaves were collected locally from neem trees at Lahore.

Phytophthora Infastans isolate KNL1, was chosen from the mycological collection of the Crop Diseases Research Institute on the basis of its aggressiveness and was maintained on rye agar media (Caten and Jinks, 1968).

Bioassays to evaluate activity of the neem products against Phytophthora infestans: The study was performed by growing the fungus on rye agar medium amended with the neem products and comparing its growth on medium without any addition. Crude neem seed oil was incorporated in the medium at 1, 2 and 3 percent (v/v). Nimbokil was used at 0.1, 0.2, 0.3, 0.4, 0.5 and 1 percent (v/v). Media without any addition served as control. For crude neem seed oil terpenoid extract stock solution was prepared by dissolving 250 mg terpenoid extract powder in one ml DMSO. From this stock, calculated volume was added to the medium so that concentrations of the terpenoid in it were 0.5, 1, 10, 50, 70 and 90 ppm. Control was prepared by adding 1.5 ml DMSO to 11 of medium. The three neem products were tested for their respective doses both with and without heat sterilization. For a products to be used with heat sterilization was added to the medium before autoclaving otherwise it was added to the autoclaved medium. Three replicates for each dose were prepared along control.

For neem leaf decoction leaves dried in shade for twelve days were powdered in a grinder, soaked overnight in distilled water at 5 gm/20 mL and filtered through whatman No. 1 filter paper. One portion of the decoction was heat sterilized along medium and another portion was added to the autoclaved medium after being filter sterilized by passing it through 0.45 μm millipore filter. The decoction concentrations incorporated in terms of leaf weight per 100 ml of the medium were 0.25 g, 0.59, 0.75 g1, 1.0 g and 1.5 g per 100 mL. Control was prepared bV adding 1.5 mL sterilized distilled water to 100 mL medium.

About 15 mL medium was poured into each of 90 mm diem petriplates and 5.5 mm dia mycelial discs from the tip of actively growing *P. infestans* culture were placed in the centre of the plate. The inoculated plates were incubated at $19+1°C$. Radial mycelial growth was measured eta three weeks.

A sporangial suspension of 5×10^4 sporangia/mL was prepared by washing 14 days old cultures in 10 mL sterilized distilled water and adjusting sporangial count with theiffelp of hemocytometer. 2 mL suspension was dispensed in sterilized screw top tubes to which neem product was added in calculated amount. Crude neem'seed oil was added at 1, 2 and 3 percent (v/v), nimbokil at 0.6, 1, 1.5 and 2 percent (v/v), crude neem terpenoid extract at Oppm (control), 50, 70 and 90 ppm and neem leaf decoction at lg, 2g, and 3g per 100 mL. For crude neem terpenoid extract and neem leaf decoction the stocks prepared in the previous assay were used in calculated amount. Sporangial suspension without any addition served as control while for crude neem seed terpenoid extract control was prepared by adding calculated amount of DMSO to

sporangial suspension. The tubes were incubated at 19 + 1°C. Percent sporangial germination was assesed by counting germinated and ungerminated sporangia under 40 × magnification of compound microscope at 24 hr interval. Successive counts were made till there was no further germination.

LD_{50} values, calculated from the results of the activity of neem products against mycelial growth, were used in this study. Separate LD_{50}s i.e., LD_{50}H for heat treated and LD_{50}H unheated neem products were calculated following Abbott (1925). The doses of neem products were added to rye agar medium before or after autoclaving, as per requirement. The medium was poured into 90 mm diem petriplates at 15 mL per plate and 5.5 mm dia mycelial discs from the tip of actively growing *P. infestans* culture were placed in the centre of the plate. The inoculated plates were incubated at 19 + 1°C. After three weeks two mycelial discs of 5.5 mm dia, one from the edge of the culture and second next to the first towards centre of the plate, were taken, smashed on microscopic slide and examined for number of sporangia under 40 × magnification of compound microscope.

The oil was diluted in HPLC-grade methanol (g) 100 mg oil/mL methanol to eliminate non polar triglycerides etc. from the oil. It was then made to 90 percent aqueous methanol by the addition of 0.1 vol. of water and partitioned with an equal volume of light petroleum. Phases were separated by centrifugation for five minutes at 1500 rpm and upper phase discarded.

Crude neem seed terpenoid extract powder and pure azadirchtine was dissolved in HPLC-grade methanol at 1 mg/ml and 100 mg/ml respectively.

The air dried neem leaves were extracted with water at 0.5 mg/100mL water by homogenization with ultra homogenizer for 30 sec. The extract was left steeping over night before removing the leaf material. Homogenate was centrifuged at 10,000 rpm for 10 minutes to separate clear supernatant.

Chromatography was performed on 25 cm reverse phase column based on a C6 spacer attached to a silica matrix. The solvent employed was a gradient of HPLC-grade methanol in water, starting at 50 percent methanol and rising to 100 percent in a linear gradient over a period of 20 minutes. Compounds were detected by their absorbance at 220 nm. Azadirchtin was quantified by measuring the area under the peak and comparing it to that of known amounts of the standard.

Results and Discussion

Crude neem seed oil significantly affected mycelial growth of *P. infestans* when used either with or without being autocraved (Table 2). It also reduced sporangial germination (Table 7) and sporangial production of the fungus (Table 6) which indicate the biologically active nature of the oil. HPLC analysis show that the oil is composed of complex mixture of presumed terpenoids of which only azadirchtin (Aza) could be identified (Fig. 1). Biological activity of the oil against *P. infestans* is probably because of this complex mixture of terpenoids most of which like azadirchtin and its derivatives are known to have biological activity against fungi like *Aspergillus fumigates* and *A. wantti* (Anonymous, 1991). The activity of the oil seems to be reduced by heat treatment, most probably because of the volatile or heat labile nature of some of the biologically active compounds. In the presence of neem oil germinated sporangial count, although less than control at 2 and 3 percent dose, increased significantly with the passage of time (Table 7) which may be due to the non availability of some of the terpenoids as most of them are of volatile nature (Wan *et al*., 1996).

Nimbokil, a commercial formulation of crude neem seed oil, was found significantly prominent in inhibiting different growth stages of *P. infestans* at much lower concentrations as compared to crude neem seed oil (Table 3, 6 and 8). On sporangial germination nimbokil seems to have a relatively stable effect and does not appear to loose its efficacy with the passage of time. Relatively higher efficacy of nimbokil is perhaps due to the presence of some stabilizers and protectants, added to this commercial formulation, which may impart stability to terpenoids in addition to having direct probable antifungal activity.

Crude neem seed oil terpenoid extract was highly effective at very low concentration i.e. 0.5 ppm against mycelial growth (Table 4), at 70 ppm against sporangial germination (Table 9) and at a 7.7 ppm against sporangial production (Table 6). Like the other two neem seed based products, unautoclaved crude neem seed oil terpenoid extract was more effective against mycelial growth and sporangial germination of *P. infestans.* Crude neem seed oil terpenoid extract although reduced sporangial production significantly, it does not seem to have produced significant difference in LD_{50}H and LD_{54}Ho (Table 6). Preliminary results of HPLC suggest that on the basis of retention time both neem seed oil and its terpeniod extract contain an almost identical range of compounds (Fig. 1 and 2). At least one compound with retention time 13.4 min which is present in oil is absent from crude neem seed terpenoid extract. Most of the presumed terpenoids could not be identified and only azadirchtin with retention time

Table 1: LD_{50} values for autoclaved and unautoclaved neem products

S. No.	neem product used	LO_{50} value for unautoclaved product (LD_{50}Ho)	LO_{50} value for autoclaved product (LD_{50}H)
1	crude neem seed oil	0.55% (v/v)	1.3% (v/v)
2	nimbokil	0.059%(v/v)	0.065% (v/v)
3	crude neem seed oil terpenoid extract	7.7 ppm	53 ppm
4	neem leaf decoction	0.16 g/100mL	0.14 g/100mL

Table 2: Effect of crude neem seed oil on mycelial growth *P. infestans*

Crude neem seed oil concentration (%)	With autoclaving		Without autoclaving	
	Myceliel growth (mm)	0.05% Confidence interval	Mycelial growth (mm)	0.05% Confidence interval
0	84.3	84.3,84.3	84.0	84.84
1	60.3	61.8,58.6	2.2	2.9,1.5
2	1.9	2.2,1.6	0.0	0.0,0.0
3	0.0	0.0,0.0	0.0	0.0,0.0

Table 3: Effect of Nimbokil on Mycelia! Growth of *P. infestans*

Nimbokil concentration (%)	With autoclaving		Without autoclaving	
	Mycelial growth (mm)	0.05% confidence interval	Mycelial growth (mm)	0.05% confidence interval
0.0	84.5	84.5, 84.5	84.5	84.5, 84.5
0.1	19.6	22.0, 17.2	8.2	8.6, 7.8
0.2	14.3	17.6, 11.0	7.0	7.7, 6.3
0.3	13.5	15.7, 11.3	6.5	6.9, 6.1
0.4	4.6	5.7, 3.5	0.0	0.0, 0.0
0.5	2.5	3.2, 1.8	0.0	0.0, 0.0
1.0	0.0	0.0, 0.0	0.0	0.0, 0.0

Table 4: Effect of Crude Neem Seed Oil Terpenoid Extract on Mycelia! Growth of *P. infestans*

Terpenoid extract concen -tration (ppm)	With autoclaving		Without autoclaving	
	Mycelial growth (mm)	0.05% confidence interval	Mycelial growth (mm)	0.05% confidence interval
0 (No. DMSO)	84.5	84.5, 84.5	84.5	84.5, 84.5
0 (DMSO)	84.5	84.5, 84.5	84.5	84.5, 84.5
0.5	84.5	84.5, 84.5	43.0	44.2, 41.8
1	84.5	84.5, 84.5	40.0	43.0, 37.0
10	84.5	84.5, 84.5	35.0	36.9, 33.1
50	42.7	44.3, 41,1	0.0	0.0, 0.0
70	34.0	34.8, 33.2	0.0	0.0, 0.0
90	28.0	30,6, 25.4	0.0	0.0, 0.0

Table 5: Effect of neem leaf decoction on mycelia' growth of *P. infestans*

Decoction Concentration Equivalent (Leaf wt. (g) per 100 mL of medium)	With Autoclaving		Without Autoclaving	
	Myceilal growth (mm)	0.05% confidence interval	Myceilal growth (mm)	0.05% confidence interval
0.	84.5	84.5,84.5	84.5	84.5,84.5
0.25	9.7	10.5,8.9	11.0	11.7,10.3
0.50	4.5	4.8,4.2	5.7	6.1,5.3
0.75	0.0	0.0,0.0	4.7	5.1,4.3
1.00	0.0	0.0,0.0	3.6	4.0,3.2
1.50	0.0	0.0,0.0	0.0	0.0,0.0

Table 6: Effect of Different Neem Products at theit respective LD5Os on Sporangial Production of *P. infestans*

S. No.	Isolate/Treatment	Sporangial Production (No.)	0.05% Confience interval
1	Control	32.3	(34.4, 30.2)
2	Crude Neem Seed Oil With autoclaving (LD_{50}H 1.3%)·	27.7	(32.1,23.3)
	Without autoclaving (LD_{50}Ho .55%)**	26.7	(30.1,23.3)
3	Nimbokil With autoclaving (LD_{50}H .65%)	20.7	(21.5,19.9)
	Without autoclaving (LD_{50}Ho .59%)	19.3	(20.0,18.6)
4	Crude Neem Seed Oil Terpenoid Extract		
	With autoclaving (LD_{50}H .0053%)	26.0	(29.6,22.4)
	Without autoclaving (LD_{50}Ho .00077%)	23.3	(24.1,22.5)
5	Neem Leaf Decoction		
	With autoclaving (LD_{50}H 14g/100 ml)	21.7	(22.4,21.0)
	Without autoclaving (LD,Ho .16g/100 ml)	22.3	(24.4,20.2)

* LD_{50} value of autoclaved product ** LD_{50} value of unautoclaved product

*** Average of sporangial number in nine microscopic field at 40x

Table 7: Effect of Crude Neem Seed Oil on Sporangial Germination of *P. infestans*

Conc. /Days	0.0%	Sporangial Germination (%)					
		1% (Autoclaving)		2% (Autoclaving)		3% (Autoclaving)	
		With	Without	With	Without	With	Without
1	40.4 (52.2,28.6)	38.5 (46.8,30.2)	34.7 (43.5,25.9)	25.2 (31.7,18.7)	19.4 (26.0,12.8)	4.2 (11.2,1.8)	3.3 (11.2,4.6)
3	38.9 (45.5,32.4)	40.0 (51.7,28.3)	38.8 145.4,32.2)	29.1 (34.8,23.4)	23.5 (32.3,14.7)	16.6 (26.5,6.7)	5.5 (18.6,7.6)
5	42.6 (49.2,35.9)	38.6 (47.6,29.5)	37.6 (47.3,27.5)	32.3 (45.3,19.3)	25.0 (25.0,25.0)	21.8 (30.5,12.9)	13.8 (16.9,10.7)
7	44.0 (48.9,39.1)	39.1 (47.1,31.1)	37.2 (46.5,27.9)	34.2 (39.0,29.0)	26.3 (29.5,23.2)	29.1 (34.8,23.4)	16,6 (16.6,16.6)

Values in parenthesis give the conifendence limit at 0.05% t level

Table 8: Effect of Nimbokil on Sporangial Germination of *P. infestans*

Conc. /Days	Sporangial Germination (%)							
	0.0%	0.5% (Autoclaving)		1.0% (Autoclaving)		1.5% (Autoclaving)		2.0% (Autoclaving)	
		With	Without	With	Without	With	Without	With	Without
1	42.9 (54.7,31.1)	12.5 (16.0,9.0)	6.6 (8,7,4.5)	8.9 (11.0,6.8)	4.1 (5.4,2.8)	3.3 (6.2,0.4)	0.0 (0.0,0.0)	0.0 (0.0,0.0)	0.0 (0.0,0.0)
3	41.4 (51.7,31.1)	15.3 (18.6,12.0)	9.6 (11.9,7.3)	10.0 (12.3,7.7)	6.6 (10.0,3.4)	6.7 (0.0,0.0)	0.0 (0.0,0.0)	0.0 (0.0,0.0)	0.0 (0.0,0.0)
5	42.8 (45.5,40.1)	18.6 (23.2,14.0)	12.6 (12.6,12.6)	12.5 (16.5,8.5)	6.6 (7.0,6.2)	8.3 (12.4,4.2)	0.0 (0.0,0.0)	0.0 (0.0,0.0)	0.0 (0.0,0.0)
7	44.0 (55.0,33.0)	20.8 (26.3,15.3)	12.5 (15.2,9.8)	14.4 (17.6,11.2)	8.3 (10.4,6.2)	8.9 (11.9,5.9)	0.0 (0.0,0.0)	0.0 (0.0,0.0)	0.0 (0.0,0.0)

* Values in the parenthesis give the confidence limit at 0.05% t level

Table 9: Effect of Crude Neem Seed Oil Terpenoid Extract on Sprangial Germination of *P. infestans*

Conc. /Days	Sporangial Germination (%)							
	0.0%	0.0% (DMSO)	.005% (Autoclaving)		.007% (Autoclaving)		.009% (Autoclaving)	
			With	Without	With	Without	With	Without
1	41.7 (48.1,35.1)	40.4 (52.2,28.6)	16.6 (26.5,6.7)	9.7 (21.6,2.2)	8.3 (18.2,1.6)	6.7 (14.6,1.2)	4.17 (14.1,5.7)	3,3 (11.2,4.6)
2	44.4 (51.0,37.8)	41.8 (57.3,26.3)	25.2 (31.7,18.7)	11.1 (24.3,2.1)	13.9 (17.1,10.8)	8.3 (18.2,1.6)	7.5 (16.8,1.8)	0.0 (0.0,0.0)
5	40.1 (43.8,36.4)	39.0 (52.3,25.7)	33.3 (43.2,23.4)	16.6 (26.5,6.7)	25.0 (25.0,25.0)	13.9 (17.4,10.4)	0.0 (0.0,0.0)	0.0 (0.0,0.0)
7	41.6 (53.1,30.1)	40.6 (44.6,36.6)	33.1 (38.9,27.3)	19.4 (35.9,2.9)	29.8 (31.8,27.8)	15.3 (18.6,12.00)	0.0 (0.0,0.0)	0.0 (0.0,0.0)

* Values in the parenthesis give the confidence limit at 0.05% t level

Table 10: Effect of neem leaf decoction on sporangial germination of *P. Infestans*

Conc. /Days	0.0g/100ml	Sporangial Germination (%)					
		1g/100ml (Autoclaving)		2g(Autoclaving) With		3g(Autoclaving)	
		With	Without	With	Without	With	Without
1	43.0 (46.5,39.5)	8.3 (18.2,1.6)	9.7 (15.6,8.2)	5.5 (18.6,7.6)	4.17 (14.1,5.7)	0.0 (0.0,0.0)	0.0 (0.0,0.0)
3	46.6 (44.3,36.9)	21.8 (37.2,6.4)	22.2 (35.4,9.4)	14.3 (19.4,9.16)	'13.9 (17.4,10.4)	0.0 (0.0,0.0)	0.0 (0.0,0.0)
5	38.0 (42.6,33.4)	33.3 (43.2,23.4)	32.0 (40.8,23.2)	23.1 (35.0,11.2)	22.7 (30.0,15.4)	0.0 (0.0,0.0)	0.0 (0.0,0.0)
7	40.8 (44.9,36.7)	34.6 (53.1,16.1)	34.7 (38.0,31.4)	25.0 (31.9,18.1)	23.6 (32.4,14.8)	0.0 (0.0,0.0)	0.0 (0.0,0.0)

* Values in parenthesis give the confidence limit at 0.05% t level

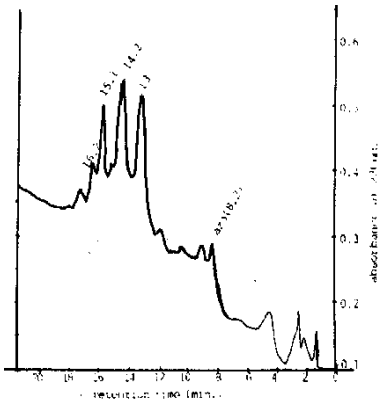

Fig. 1: Resolution of terpenoids in neem oil by reverse Phase HPLC

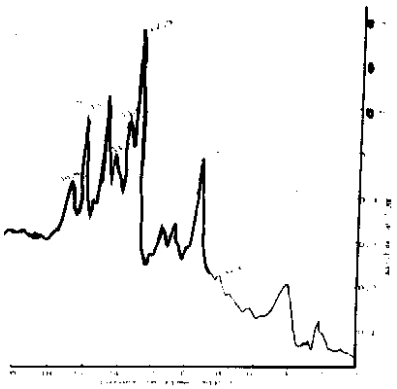

Fig. 2: Resolution of terpenois in crude neem seed terpenoid extract by reverse phase HPLC

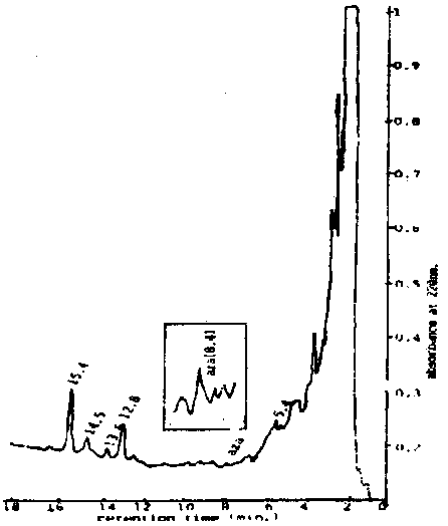

Fig. 3: Resolution of compounds extracted from neem leaves with water by reverse phase HPLC

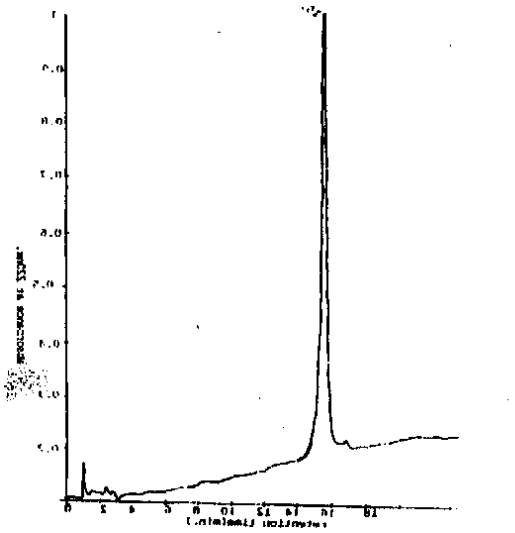

Fig. 4: Retntion time of a pure sample of gedunin by reverse phase HPLC

8-8.4 min could be identified (Fig. 1 and 2). Concentration of terpenoids in the oil is much lower as compared to that in crude neem seed oil terpenoid extract, eg. on weight for weight basis concentration of azadirrchtin was estimates as 100 times lower in oil as compared to crude neem seed oil terpenoid extract. This explains significant effect of the crude neem seed oil terpenoid extract at very low doses and confirm the activity of the terpenoids in oil and nimbokil against mycelia! growth, sporangial germination and sporangial production of *P. infestans.*

Autoclaved and unautoclaved neem leaf decoction was equally effective against the different growth stages of *P. infestans* without much significant difference (Table 5, 6 and 10), In addition to commonly found unidentified compounds with retention time 12.8, 13.6, 14.5 and 5.4 minutes, Gedunin with retention time 15.4 minutes, is the major terepenoid found in leaves (Fig 3 and 4). Gedunin, the thermostable nature of which is well reported (Sadre *et al*., 1983), seems to be responsible for antifungal activity of the decoction which did not loose its activity by heat treatment.

Comparatively lower $LD_{50}H$ values (Table 1) for neem seed oil based products clearly indicate that all the products have common heat labile biologically active ingredients. Relatively higher value of $LD_{50}H$ as compared to $LD_{50}Ho$ (Table 1) shows the thermally stable nature of its biologically active ingredient. From these studies it become clear that leaf originated products retain their activity after autoclaving and are thermostable where as the activity of the neem seed oil based products is reduced after autoclaving. This is primarily due to difference in principle active ingredients of these products. However, nimbokil, being a formulated product of crude neem seed oil, was relatively more effective than other neem seed oil based products probably because of the presence of stabilizer and protectants which appear to have antifungal activity.

The present study has opened the scope for further research work on biochemistry to identify the antimycotic compounds of neem which may further be exploited for the control of potato late blight diseases.

Acknowledgements

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