INDIAN INSTITUTE OF SPICES RESEARCH
KOZHIKODE-673-012, KERALA
(Indian Council of Agricultural Research)

FINAL RESEARCH PROJECT REPORT (RPP- III)

1. Institute Project Code : Nema IV (813)

2. Project Title : **ROLE OF PHENYL PROPANOIDS IN BLACK PEPPER – BURROWING NEMATODE INTERACTIONS**

3. Key Words : Black pepper, burrowing nematode, host-parasite interaction, phenylpropanoids, *Piper nigrum*, *Radopholus similis*

4. (a) Name of Lead Institute : ICAR-Indian Institute of Spices Research
(b) Name of Division/Regional Center/Section : Division of Crop Protection

5. (a) Name of the Collaborating Institute(s) : Nil
(b) Name of Division/Regional Center/Section of Collaborating Institute(s) : NA

6. Project Team (Name(s) and designation of PI, CC-PI and Co-PIs, with time spent :

Sl. No.	Name, designation and institute	Status in the project (PI/CC-PI/ Co-PI)	Time spent (%)	Work components assigned to individual scientist
1.	Santhosh J. Eapen Principal Scientist	PI	50	• Studies on cell wall degrading enzymes secreted by *R. similis* • Bioassay of selected compounds for nematode inhibition
2.	A. Shamina, Senior Scientist (up to 2012)	Co-PI	40	• Monitoring changes in enzyme activities • Extraction and quantification of phenylpropanoids • Studies on cell wall degrading enzymes secreted by *R. similis*
3.	Johnson K. George, Principal Scientist (from 2013 onwards)	Co-PI	10	-

7. Priority Area : Basic Research

8. Project Duration: Date of Start : 2008 Date of Completion: 2014

9. (a) Objectives:

a. To study the post-infectional changes in phenylpropanoid pathway in black pepper infested with *Radopholus similis*
b. To characterize defence responses to *R. similis* in susceptible and resistant black pepper lines
c. To investigate the inhibitory effects of phenylpropanoid pathway products on plant parasitic nematodes

(b) Practical Utility:

Immediate benefits: A better understanding of the plant-nematode interaction is urgently needed for evolving an effective management strategy against *R. similis* in black pepper nurseries and plantations. The proposed study is envisaged to elucidate the nature of resistance in some of the identified black pepper cultivars too.

Medium term benefits: The natural compounds that are identified as nematode inhibitory or repellent can be scaled up as commercial nematicides. The gene transcripts of such compounds that can be extended further to identification, cloning and sequencing of genes involved in parasitism and other nematode specific processes.

Long term benefits: The identification of genes involved in nematode parasitism as well as the utilization of nematode inducible plant genes, will be valuable resources for creating new forms of durable plant resistance, and may lead to genetic engineering for nematode resistance in important cultivars of black pepper.

10. Final Report of the Project Please see Annexure 1

(a) Materials and Methods:

(b) Results and Discussion:

(c) Objective-wise Achievements:

(b) Conclusions:

11. Financial Implications (Lakh Rs.):

11.1 Expenditure on

(a) Manpower	:	20.78
(b) Research/Recurring Contingencies	:	1.25
(c) Non-Recurring Cost (Including Cost of Equipment)	:	0.00
(d) Any Other Expenditure Incurred	:	0.00
11.2 Total Expenditure	:	22.03

12. Cumulative Output:

(a) Special Attainments/Innovations:

- Salicylic acid, syringaldehyde and ferulic acid were found to possess potential nematicidal activity under in vitro and greenhouse assays which has to be further validated through extensive pot/field trials.
- Higher levels of proanthocynidins in black pepper roots is an indicator of nematode resistance and can be developed as a good technique for screening for resistance.

(b) List of publications (1 copy each to be submitted):

i. Research Papers :

- Sangeetha K., Rosana, O.B. and Eapen, S.J. 2012. EST secretome analysis of *Radopholus similis* nematode. *Online J. Bioinformatics* 13 (1):103-119.
- Rosana OB, Krishna PB and Eapen SJ 2014. Virtual screening and in vitro assay to explore novel inhibitors from black pepper against potential targets of *Radopholus similis*. *International Journal of Computer Applications* 86(14): 35-43.

ii. Reports/Manuals : Nil

iii. Working and Concept Papers : Nil

iv. Popular Articles : Nil

v. Books/Book Chapters :

- Eapen, S.J. 2010. Nematode infestation in spice cultivations. In: *Nematode Infestations Part II: Industrial Crops*. Pp. 82-119. Eds. M.R. Khan and M.S. Jairajpuri. The National Academy of Sciences, Allahabad, India.
- Suseela Bhai, R. and Eapen S.J. 2010. Integrated management of diseases of important spice crops. In: *Sustainable Crop Protection Strategies* Vol. I. Pp. 282-333. Eds. H.R. Sardana, O.M. Bambawale and D. Prasad. Daya Publishing House, New Delhi.
- Sirohi, A., Nagesh, M. and Eapen S.J. 2012. Application of molecular approaches in nematode biocontrol. In: *Status and Prospects for enhancing the Uptake of Antagonistic Organisms for Nematode Management in India*. Pp. 134-146, Eds. M. Nagesh, Rajkumar, B.S. Bhumannavar & N.K. Krishna Kumar. National Bureau of Agriculturally Important Insects, Bangalore, Karnataka.

vi. Extension Bulletins : Nil

(c). Intellectual Property Generation (Patents: filed/obtained; Copyrights filed/obtained; Designs: filed/ obtained; Registration details of variety/germplasm/accession) : Nil

(d) Presentation in Workshop/Seminars/Symposia/Conferences (relevant to the project):

- Sangeetha, K., Riju, A., Rosana, O.B., Chandrasekar, A., Reena, N. and Eapen, S.J. (2011). EST based secretome analysis of burrowing nematode, *Radopholus similis*. Silver Jubilee Symposium on Bioinformatics. Centre for Bioinformatics, Pondicherry University, Pondicherry.

- Rosana OB, Dinsha M, Shamina A and Eapen S J (2011). *In silico* and *in vitro* studies to explore potential nematicidal phenylpropanoids from *Piper nigrum* L. against *Radopholus similis*. Presented in International Symposium on Biocomputing on 12-13 September 2011, Calicut, Kerala, India. Poster No: ISBPS050.
- Santhosh J Eapen, Krishna P B, Shamina A and R R Nair (2014). Biochemical responses of black pepper to infestation by burrowing nematode, *Radopholus similis*. In: Abstracts-PLACROSYM XXI, International Symposium on Plantation Crops, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India.

(e) Details of technology developed (Crop-based; Animal-based, including vaccines; Biological-biofertilizer, biopesticide, etc; IT based-database, software; Any other-please specify):

- Salicylic acid, syringaldehyde and ferulic acid were found to possess potential nematicidal activity under *in vitro* and greenhouse assays which has to be further validated through extensive pot/field trials.
- Higher levels of proanthocynidins in black pepper roots is an indicator of nematode resistance and can be developed as a good technique for screening for resistance.

(f) Trainings/demonstrations organized: Nil

(g) Trainings received : Nil

(h) Any other relevant information : Nil

13. (a) Extent of achievement of objectives and outputs earmarked as per RPP-I:

Objective wise	Activity	Envisaged output of monitorable target(s)	Output achieved	Extent of achievement (%)
1.	1. Studies on changes in phenylpropanoid pathway enzymes consequent to *R. similis* infection	Monitoring changes in enzyme activities - Phenylalanine ammonia lyase (PAL) - Cinnamic acid-4-hydrolase (C4H) - Caffeic acid–O-methyltransferase (COMT)	Changes in all the three phenyl propanoid pathway enzymes viz. PAL, C4H and COMT were monitored in a susceptible (Sreekara) and resistant (HP 39) black pepper varieties consequent to *R. similis* infection.	100
2.	1. Biochemical changes in defense pathways in resistant vis-a-vis susceptible black pepper lines infected with *R. similis*	Extraction and quantification of phenylpropanoids like lignin, hydroxy cinnamic acid, ferulic acid and dopamine	Lignin content of the roots of the susceptible Sreekara and resistant HP-39 were estimated. However, attempts to extract and purify hydroxy cinnamic acid were not successful.	60

	2. Isolation and purification of selected products in shikimic acid pathway	Quantitative assays for anthocayanidins, proanthocyanidins and their precursors, leucoanthocyanidins	Qualitative and quantitative assays were carried out for these biochemical products in root extracts of Sreekara and HP 39	90
3.	Studies on cell wall degrading enzymes secreted by *R. similis*	Studies on cell wall degrading enzymes secreted by R. similis viz. β-1,4 endoglucanase, chorismate mutase and polygalacturonase	An EST based secretome analysis was carried out and the excretory-secretory (ES) proteins were identified. The presence of cellulose degrading enzymes was identified through some preliminary assays. Eight novel targets of *R. similis*, involved in invasion, parasitism, growth and survival were selected and modelled for further in silico analyses.	80
4.	Bioassay of selected compounds/enzymes for suppression of *R. similis*	*In silico* bioassays	Eighteen compounds out of 26 phytochemicals possessed antihelminthic (nematicidal) activity based on PASS prediction and were docked with the above eight nematode targets.	100
		In vitro bioassays	Eight promising compounds were tested for nematode mortality in four concentrations (200, 100, 50, 20 µg ml^{-1}) under laboratory conditions	100
		In planta bioassays	The best two compounds, salicylic acid and ferulic acid were tested for suppression of R. similis in a pot study.	100

(b) Reasons of shortfall, if any:

Because of the transfer of co-investigator mid-way, some of the biochemical works could not be taken up.

14. Efforts made for commercialization/technology transfer :

More studies are needed before venturing into commercialization/technology transfer.

15. (a) How the output is proposed to be utilized?

- The present screening methodology can be modified in view of the research finding that higher levels of proanthocynidins in black pepper roots is an indicator of nematode resistance.
- The natural compounds that are identified as nematode inhibitory or repellent can be used for management of nematodes after laying out further testing under field conditions.
- More *in vitro* and *in planta* assays are currently being undertaken as part of the doctoral studies of one of the student.

(b) How it will help in knowledge creation?

- The study is the first of its kind to understand the biochemical changes in black pepper consequent to nematode parasitism. It also thrown some light on the differential reaction of nematode resistant susceptible black pepper cultivars to *R. similis.*

16. Expected benefits and economic impact (if any):

- The project provided a better understanding of the plant-nematode interaction which is quite helpful for evolving an effective management strategy against *R. similis* in black pepper nurseries and plantations. Knowledge of resistance mechanism(s) in these plants could provide 'markers' to facilitate and speed up the screening of *Piper* germplasm and improved hybrids.

17. Future line of research work/other identifiable problems:

- Field evaluation of ferulic acid, syringaldehyde and salicylic acid for management of plant parasitic nematodes.
- Gene expression studies in *R. similis* exposed to above compounds
- Differential display of genes in black pepper infected with *R. similis*
- Transcriptome studies in black pepper lines resistant/susceptible to *R. similis*
- Engineering for nematode resistance in important cultivars of black pepper

18. Details of research data (registers and records) of the project deposited with the institute:

Field/Lab Observation Book No. 138

Experimental Data Register No. 111

(Both are maintained in Nematology Laboratory, ICAR-IISR, Kozhikode)

19. Signature of PI, CC-PI, Co-PIs:

Dr. Santhosh J. Eapen

Dr. Johnson K. George

20. Signature of Head of Division:

21. Observations of PME Cell based on Evaluation of Research Project after completion:

22. Signature (with comments if any along with rating of the project in the scale of 1 to 10 on the overall quality of the work) of Director :

1. *Role of phenyl propanoids in black pepper - burrowing nematode interactions*

Changes in three phenyl propanoid pathway enzymes viz. Phenylalanine ammonia lyase (PAL), cinnamic acid-4-hydrolase (C4H) and caffeic acid-O-methyltransferase (COMT) were monitored in a susceptible (Sreekara) and resistant (HP 39) black pepper varieties consequent to *R. similis* infection. For this, rooted cuttings (3 months old) were raised in sterile potting mixture in greenhouse at IISR. Cultures of *R. similis* originally isolated from black pepper was maintained axenically on carrot discs. Nematodes were collected in sterile water from six-week old carrot disks. The suspension was made up with sterile water to a concentration of 30-50 nematodes per mL. The nematode suspension (10-15 mL) was inoculated around the root zone in the adhering soil @ ~500 nematodes per plant. The inoculated plants were sampled at 48 h, 1 week and 1 month. Plants wounded at root, by trimming root tips using sterile blade and sampled at 48 h, 1 week and 1 month were used as a check while uninoculated IISR Sreekara and tolerant HP39 plants were used as controls. The samples were assayed for the activity of PAL by the method of Hahlbrock et al. (1971) and Zucker (1975) spectrophotometrically, and expressed as µM t-cinnamic acid formed per min per mL crude enzyme extract. C4H activity was quantified using a HPLC (Peterson, 2003), and expressed as µM p-coumaric acid formed per min per mL crude enzyme extract. COMT was monitored by TLC of the product followed by quantification using a spectrophotometer (Shimada et al., 1970), and expressed as µM ferulic acid formed per min per mL crude enzyme extract. Two replications were maintained for all the treatments.

The PAL activity was constitutively high in HP 39 compared to the susceptible line. However, PAL activity increased in susceptible plants immediately after wounding or infestation by *R. similis* and subsequently reduced over a period of 30 days. However, *R. similis* infestation lowered PAL activity in the resistant plants. There was not much variation in the constitutive activity of C4H in susceptible as well as resistant black pepper plants. But on infestation with *R. similis*, the activity of C4H shot up in HP 39 plants (Table 1). The changes in C4H activity were only marginal in wounded samples. The activity declined by one month after inoculation in both cases. However, in the susceptible Sreekara there were no discernible changes in the enzyme activity.

COMT activity increased within 48 h after *R.similis* inoculation/wounding in both Sreekara and in HP-39, compared to the activity in control plants. Its activity decreased thereafter.

2. *Estimation of phenyl propanoids*

Lignin content of the roots of the susceptible Sreekara and resistant HP-39 were estimated at 48 h, 7 days and a month after inoculation/wounding. Lignin content was comparatively low in HP 39 compared to Sreekara. However, it decreased significantly (by a third of the control values) in Sreekara within 48 hours of inoculation/wounding and further increased to control values in a week and again decreased in a month. In HP-39, which had only 1/3rd the lignin content of the susceptible variety in the control plants, the decrease in lignin content (by half original lignin content) continued up to the 1st week after inoculation/ wounding, and increased to double the control values in a month in the inoculated samples.

Methods to extract and purify hydroxy cinnamic acids were standardized. However, attempts to extract and purify hydroxy cinnamic acid from different sets of inoculated and control samples were not successful. Repeated attempts to extract and purify hydroxy cinnamic acid from different sets of inoculated and control samples were not successful. The work will be taken up again after modifying the protocol being used.

Table 1. Changes in the activities of phenylalanine ammonia lyase and cinnamic acid-4-hydrolase enzymes in susceptible (Sreekara) and resistant (HP 39) black pepper lines in response to *R. similis* infestation and wounding.

Treatment	**Resistant line (HP 39)**				**Susceptible line (Sreekara)**				**Gen. Mean**
	2d	7d	30d	Mean	2d	7d	30d	Mean	
Activity of Phenylalanine Ammonia Lyase (PAL) – units x 10^{-3}									
Control	18.65b	18.53c	18.58b	18.59b	6.08a	6.08a	6.08	6.08a	12.34b
R.similis inoculated	12.54a	8.78a	1.70a	7.67a	12.97b	12.05b	11.79a	12.27b	9.97a
Wounded	22.89b	14.97b	17.75b	18.54b	21.00c	9.03b	11.16a	13.73b	16.13c
Mean	18.03	14.09	12.67	14.93	13.35	9.05	9.68	10.69	-
LSD variety – NS ; LSD variety x interval – 2.32									
Activity of cinnamic acid-4-hydrolase (C4H) - units x 10^{-3}									
Control	0.93a	0.93	0.93	0.93ab	0.98a	0.98	0.98	0.98a	0.95b
R.similis inoculated	1.57a	2.49b	0.66a	1.57b	0.84a	3.80b	0.00	1.54b	1.56c
Wounded	1.07a	1.11a	0.60a	0.93a	0.64a	0.60a	0.24	0.50a	0.71a
Mean	1.19	1.51	0.73	1.14	0.82	1.79	0.41	1.01	-
LSD variety – NS ; LSD variety x interval – 0.33									

Qualitative and quantitative assays were carried out for anthocayanidins, proanthocyanidins and their precursors, leucoanthocyanidins, in root extracts by specific extraction procedures and butanol/HCl assay. Levels of leucoanthocyanidins are found to be higher in the susceptible cultivar (Sreekara) while levels of condensed tannins (proanthocyanidins and anthocyanidins) and phenols were higher in the resistant line, Hp 39, indicative of providing defense against the entry of *R. similis* to black pepper root systems (Fig. 1). The nematode infection increased the total phenol and proanthocyanidin content of susceptible and tolerant black pepper plants. Butanol/HCl assay was carried out in a few more selected lines of black pepper, reported as resistant to plant parasitic nematodes. The results have indicated that the level of proanthocynidins was comparatively high in resistant lines viz. Acc. 3219, C.1090 and *P. colubrinum* compared to susceptible lines like Sreekara or Panniyur 1.

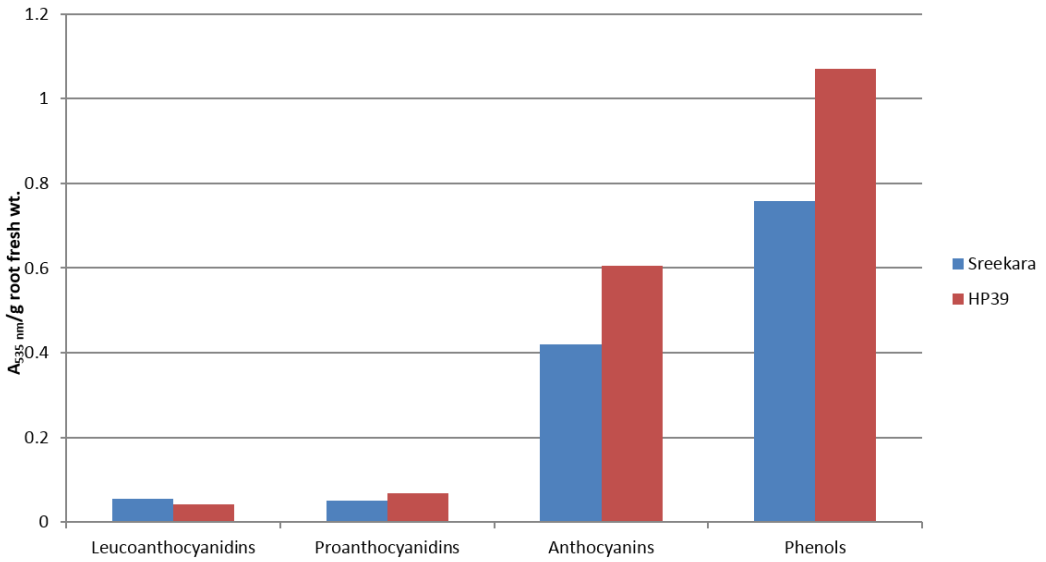


Fig. 1. Comparative levels of phenyl propanoids in black pepper lines, Sreekara and Hp 39

3. *Studies on cell wall degrading enzymes secreted by R. similis*

An EST based secretome analysis was carried out to identify the excretory-secretory (ES) proteins of *Radopholus similis*. Out of the 214 secretory proteins identified, about 45% showed similarity to proteins from other nematodes. Functional annotation of these proteins revealed the presence of some of the major secretory and cell wall degrading enzymes like transthyretin (Contig1094), GHF5 endo1, 4 beta glucanase (GW 395922), glutathione-S-transferase1 (GST) (Contig 931), glutamate dehydrogenase (Contig 395) and 3-hydroxyacyl-CoA dehydrogenase (EY193427). Total homogenates of *R. similis* showed clear cellulase activity in the CMC plate assay, and was compared with cellulase activity of *Bacillus amyloliquefaciens.*

Preliminary assays indicated the presence of cellulose degrading enzymes in burrowing nematodes.

4. *In silico screening of phenyl propanoids*

Novel targets in *R. similis*: Eight novel targets of *R. similis*, involved in invasion, parasitism, growth and survival have been used in this study, which include all possible targets of *R. similis* based on the availability of sequence and template structure information for modelling. They are β-1, 4, endoglucanase, calreticulin-1, xylanase, cathepsin B-like cysteine proteinase, cathepsin S-like cysteine proteinase, cytochrome c-oxidase subunit III, glutathione S-transferase and transthyretin-like protein 3 precursor. The selected targets and their importance in nematode survival are listed in Table 2.

Table 2. Selected targets in *R. similis* (based on available sequence data) and their importance in nematode

Target Proteins from *R. similis*	**Importance in parasitism/ survival**
Calreticulin-1	Calreticulin/calregulin is a high-capacity calcium-binding protein which is present in most tissues of nematode, they have multiple functions including regulation of signalling and metabolic pathways and controlling cell cycle progress
β-1, 4, *endoglucanase*	They are cell wall degrading enzymes which are necessary for nematodes during invasion in to host plant and plays an important role in infection and parasitism of plants
Xylanase	They are capable of hydrolyzing substituted xylan polymers into fragments of random size, functions of these enzymes are associated with a variety of developmental processes
Cathepsin B-like cysteine proteinase	Cathepsin cysteine proteinases are involved in a variety of important biological processes and have been implicated in molting, intestinal digestion and tissue remodeling in free living and parasitic nematodes; and is associated with larval molting and cuticle and eggshell remodeling
Cathepsin S-like cysteine proteinase	
Cytochrome c oxidase subunit III	They controls the last step of food oxidation and catalyze ATP synthesis
Glutathione S-transferase	Multifunctional dimeric enzymes involved in the metabolization of a broad variety of xenobiotics and reactive endogenous compounds
Transthyretin-like protein 3 precursor	They are one of the most abundant nematode-specific domains, nematode-specific nature of this gene family makes it a promising target for nematicides even though their functional role is not correctly identified [60- 61].

The side chains and hydrogen atoms were added for refining the structure and the stability of homology model has been validated by checking the geometry using PROCHECK. The model was validated using Ramachandran plot. Ramachandran plot

was identified by Procheck program of Structural Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/) and RAMPAGE- Ramachandran Plot Analysis server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). The statistics of non-bonded interactions between different atom types were detected and value of error function was analyzed by Verify_3D and ERRAT to identify overall quality factor of the model. Active site residues of modeled targets were predicted using CASTp server (http://sts-fw.bioengr.uic.edu/castp/calculation.php).

Molecular modelling of targets: Since experimentally and computationally solved structures were not available for all the selected potential targets, molecular modeling was done by using Modeller9.10 software and I-Tasser tool. Target sequences were retrieved from NCBI database. Conserved motifs present in the selected template sequences were analyzed using Conserved Domain Database (CDD) search. The sequences were then used to Blast all the protein sequences of available PDB structures. Template structures were downloaded from RCSB PDB (http://www.rcsb.org/pdb/home/home.do).

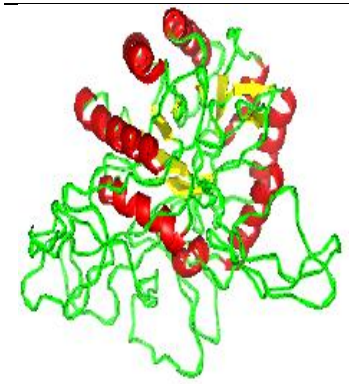
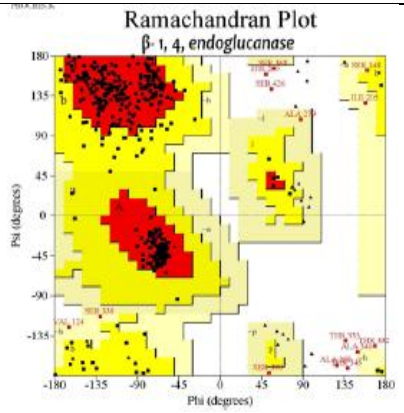
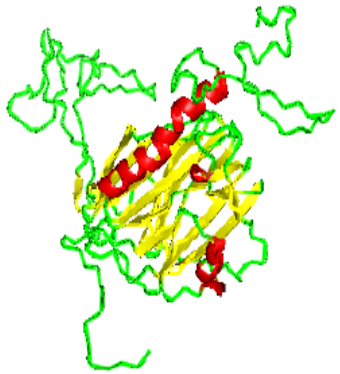
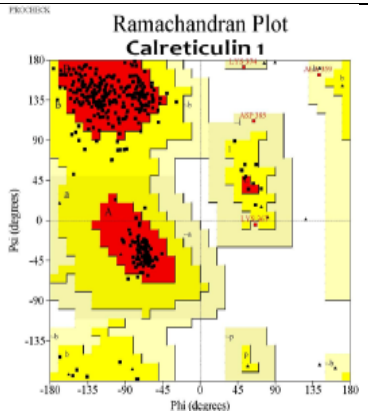
The side chains and hydrogen atoms were added for refining the structure and the stability of homology model has been validated by checking the geometry using PROCHECK. The model was validated using Ramachandran plot. Ramachandran plot was identified by Procheck program of Structural Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/) and RAMPAGE- Ramachandran Plot Analysis server (http://mordred.bioc.cam.ac.uk/~rapper/ rampage.php). The statistics of non-bonded interactions between different atom types were detected and value of error function was analyzed by Verify_3D and ERRAT to identify overall quality factor of the model. Active site residues of modeled targets were predicted using CASTp server (http://sts-fw.bioengr.uic.edu/castp/calculation.php).

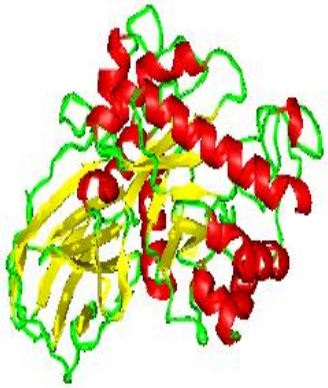
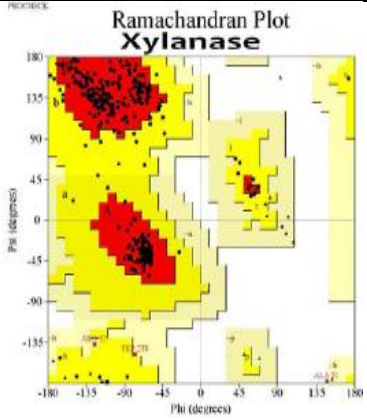
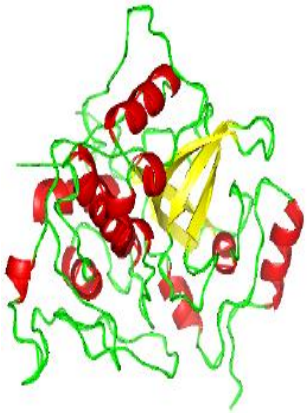
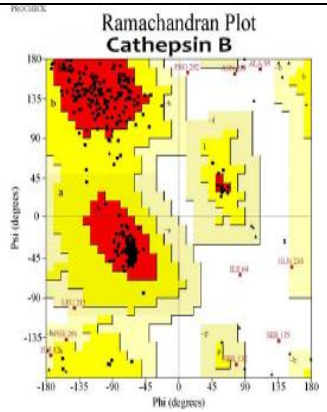
The template sequences were confirmed with the presence of conserved domain corresponding to each target using CDD search. The structures with highest identity and sequence coverage were used as template to build the 3D structures of target proteins, details of template structures and percentage identity are listed in Table 3. Three dimensional structures of modeled protein, corresponding Ramachandran plot, ERRAT confidence value, overall quality factor of modeled proteins are displayed in Table 4.

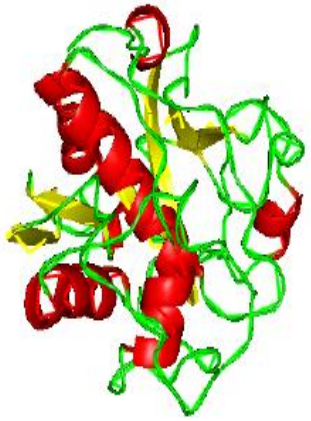
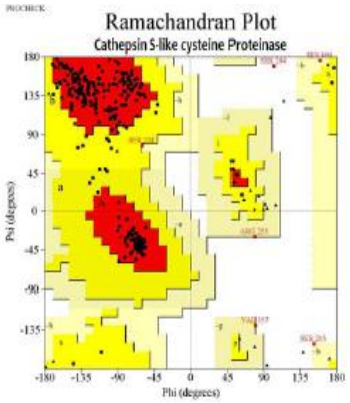
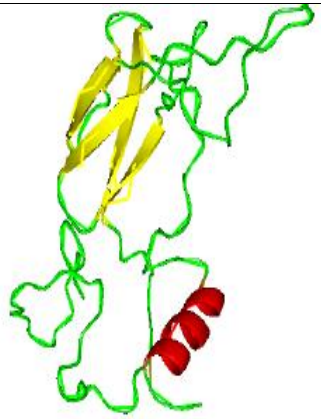
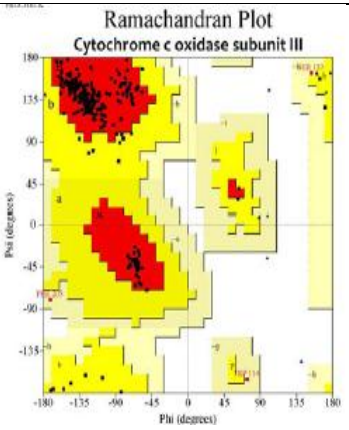
Table 3. Details of R. *similis* target sequences and template structures used for modeling

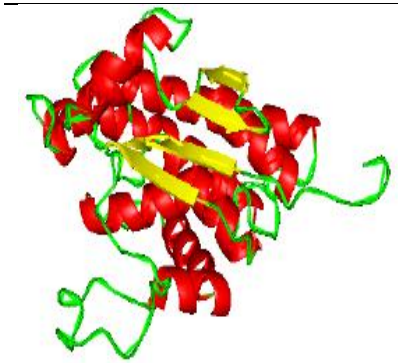
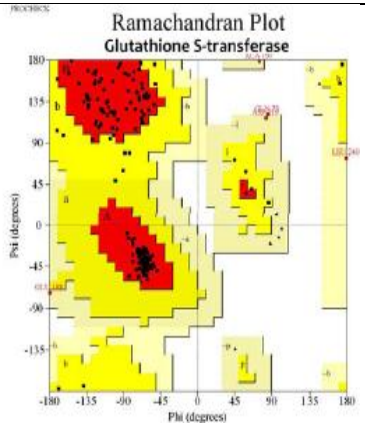
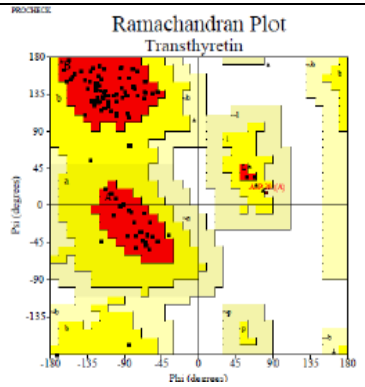
Target Sequence	Template Structure (PDB id)	X-RAY Resolution	Organism	Percentage Identity	Tool Used
β-1, 4, *endoglucanase*					
ACB38289.1	1egz Chain: A	2.30	*Erwinia chrysanthemi*	62%	Modeller9.10
	3pzv Chain: B	1.75	*Bacillus subtilis subsp. subtilis str. 168*	57%	
	1qi2 Chain: A	2.87	*Bacillus agaradhaerens*	57%	
Calreticulin-1					
ACY01917.1	3rg0 Chain: A	2.57	*Mus musculus*	65%	Modeller9.10
	3pos Chain: C	1.65	*Homo sapiens*	81%	
	3o0w Chain: A	1.95	*Mus musculus*	81%	
Xylanase					
ABZ78968.1	2y24 Chain: A	1.39	*Erwinia chrysanthemi*	64%	Modeller9.10
	3kl0 Chain: A	1.64	*Bacillus subtilis subsp. subtilis str. 168*	58%	
	3kl3 Chain: D	2.33	*Bacillus subtilis subsp. subtilis str. 168*	58%	
Cathepsin B-like cysteine proteinase					
ADK46902.1	3s3q Chain: A	1.80	*Schistosoma manson*	63%	Modeller9.10
	3pbh Chain: A	2.50	*Homo sapiens*	57%	
	3s3r Chain: B	2.64	*Schistosoma mansoni*	62%	
Cathepsin S-like cysteine proteinase					
ACH56227.1	3hwn Chain: D	2.33	*Homo sapiens*	70%	Modeller9.10
	2xu4 Chain: A	1.12	*Homo sapiens*	69%	
	2xu1 Chain: D	1.45	*Homo sapiens*	70%	
Cytochrome c oxidase subunit III					
YP_003667894.1	2ybb Chain	19.0	*Thermus thermophilus HB8*	57%	Modeller9.10
	2occ Chain: C	2.30	*Bos Taurus*	57%	
	2dys Chain: C	2.20	*Bos Taurus*	57%	
Glutathione S-transferase					
Nematode.Net: Accession: RS07755	2ws2 Chain: B	2.01	*Haemonchus contortus*	56%	Modeller9.10
	2on7 Chain: A	2.40	*Necator americanus*	54%	
	2on5 Chain: H	1.90	*Necator americanus*	57%	
Transthyretin-like protein 3 precursor					
CAM84510.1	I-TASSER server provides five best models for each protein; the best protein structure was selected on the basis of confident result obtained from I-TASSER server.				I-TASSER server

Table 4. Three dimensional structures, corresponding Ramachandran plot, ERRAT confidence value and overall quality factor of modeled proteins

β-1, 4, endoglucanase		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions: 378, (89.95%) Residues in additional allowed regions: 28, (6.65%) Residues in generously allowed regions: 6, (1.4%) Residues in disallowed regions: 8, (1.9%) ---- ------ Number of non-glycine and non-proline residues 420, (100.0%) Number of end-residues (excl. Gly and Pro) 2 Number of glycine residues (shown as triangles) 34 Number of proline residues 11 ---- Total number of residues 467 Verify_3D: 68.25% of the residues had an averaged 3D-1D score > 0.2 ERRAT: overall quality factor: 82.389
Calreticulin 1		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions 337, (93.6%) Residues in additional allowed regions 19, (5.3%) Residues in generously allowed regions 4, (1.1%) Residues in disallowed regions 0 (0.0%) ---- ------ Number of non-glycine and non-proline residues 360, (100.0%) Number of end-residues (excl. Gly and Pro) 2 Number of glycine residues (shown as triangles) 21 Number of proline residues 22 ---- Total number of residues 405 Verify_3D: 79.28% of the residues had an averaged 3D-1D score > 0.2 ERRAT: overall quality factor: 88.956

Xylanase		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions 330, (93.8%) Residues in additional allowed regions 19, (5.4%) Residues in generously allowed regions 2 (0.6%) Residues in disallowed regions 1 (0.3%) ---- ------ Number of non-glycine and non-proline residues 352 (100.0%) Number of end-residues (excl. Gly and Pro) 2 Number of glycine residues (shown as triangles) 33 Number of proline residues 13 ---- Total number of residues 400 Verify_3D: 69.54% of the residues had an averaged 3D-1D score > 0.2 ERRAT: overall quality factor: 79.634
Cathepsin B like cysteine proteinase		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions 270, (92.2%) Residues in additional allowed regions 14, (4.8%) Residues in generously allowed regions 5, (1.7%) Residues in disallowed regions 4, (1.4%) ---- ------ Number of non-glycine and non-proline residues 293 (100.0%) Number of end-residues (excl. Gly and Pro) 12 Number of glycine residues (shown as triangles) 29 Number of proline residues 18 ---- Total number of residues 352 Verify_3D: 65.33% of the residues had an averaged 3D-1D score > 0.2 ERRAT: overall quality factor: 73.734

Cathepsin S-like cysteine proteinase		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions 256, (92.4%) Residues in additional allowed regions 15, (5.5%) Residues in generously allowed regions 5, (1.8%) Residues in disallowed regions 1, (0.4%) ---- ------ Number of non-glycine and non-proline residues 277 (100.0%) Number of end-residues (excl. Gly and Pro) 2 Number of glycine residues (shown as triangles) 27 Number of proline residues 8 ---- Total number of residues 314 Verify_3D: 79.69% of the residues had an averaged 3D-1D score > 0.2 ERRAT: overall quality factor: 82.115
Cytochrome c oxidase subunit III		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions 221, (94.1%) Residues in additional allowed regions 11, (4.7%) Residues in generously allowed regions 3, (1.3%) Residues in disallowed regions 0, (0.0%) ---- ------ Number of non-glycine and non-proline residues 235, (100.0%) Number of end-residues (excl. Gly and Pro) 2 Number of glycine residues (shown as triangles) 14 Number of proline residues 6 ---- Total number of residues 257 Verify_3D: 79.56% of the residues had an averaged 3D-1D score > 0.2 ERRAT: overall quality factor: 70.152

Glutathione **S-transferase**		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions 217, (97%) Residues in additional allowed regions 2, (0.9%) Residues in generously allowed regions 2, (0.9%) Residues in disallowed regions 1, (0.5%) ---- ------ Number of non-glycine and non-proline residues 222 (100.0%) Number of end-residues (excl. Gly and Pro) 2 Number of glycine residues (shown as triangles) 10 Number of proline residues 13 ---- Total number of residues 247 Verify_3D: 75. 98% of the residues had an averaged 3D-1D score > 0.2 ERRAT: overall quality factor: 65.729
Transthyretin-like protein 3 precursor		
MODELLED PROTEIN	**RAMACHANDRAN PLOT**	**QUALITY PARAMETERS**
		Residues in most favored regions 123, (92.0%) Residues in additional allowed regions 7, (5.3%) Residues in generously allowed regions 3, (2.3%) Residues in disallowed regions 0, (0.0%) ------ Number of non-glycine and non-proline residues 133, (100.0%) Number of end-residues (excl. Gly and Pro) 2 Number of glycine residues (shown as triangles) 14 Number of proline residues 9 ---- Total number of residues 158 Verify_3D: 96.58% of the residues had an averaged 3D-1D score > 0.2 ERRAT: Overall quality factor 89.720

Ligand Structure: The 3D structures of compounds were developed by ACD/ChemSketch Version 10.0 for Microsoft Windows using canonical smiles of the compounds collected from PubChem (http://pubchem.ncbi.nlm.nih.gov/). Energy minimization and molecular optimization of the compounds were done using Arguslab 4.0.1. Geometry optimization was carried out using AM1 (Austin Model 1), semi-empirical quantum mechanics force field in Arguslab4.0.1. The best conformer exhibiting lowest energy thus obtained based on energy minimization and geometry optimization were saved in *.pdb format for input into docking environment.

PASS prediction: The compounds of phenylpropanoid metabolic pathway from black pepper have been screened for activities such as antihelminthic (nematicidal) activity in PASS (Predicted Activity Spectrum of Small Molecules) server (http://www.pharmaexpert.ru/passonline/). PASS predicts over 4000 kinds of biological activity, including pharmacological effects, mechanisms of action, toxic and adverse effects, interaction with metabolic enzymes and transporters, influence on gene expression, etc., on the basis of structural formula of a substance. PASS prediction gives out a list of activities with appropriate Pa and Pi, sorted in descending order of difference (Pa-Pi)>0. *Pa (probability "to be active")* estimates the chance that the studied compound is belonging to the sub-class of active compounds, *Pi (probability "to be inactive")* estimates the chance that the studied compound is belonging to the sub-class of inactive compounds based on resemblance with structures of the molecules, which are the most typical in a sub-set of "active and inactive" in PASS training set. For each type of activity of the biological activity spectrum; Pa-Pi values vary from 0.000 to 1.000. As per PASS prediction of biological activity, if the value of Pa>0.5 in a scale of 0 to 1 unit, the compound is expected to reveal the activity in experiments. If Pa>0.7, the compound is very likely to exhibit the activity in wet lab experiments. The compounds which showed selected activity in PASS screening have been taken as ligands for the studies.

PASS biological activity prediction for compounds in phenyl propanoid pathway revealed that 18 compounds out of 26 phytochemicals possess antihelminthic (nematicidal) activity based on predicted Pa and Pi values. PASS screening results for 18 compounds with predicted Pa; Pi values for antihelmintic property are shown in Table 5.

Table 5. Pa and Pi values for antihelmintic property in eighteen phytochemicals predicted through PASS bioactivity screening

Compound	Predicted Activity: Antihelmintic	
	Pa	Pi
syringaldehyde	0.993	0.004
syringin	0.984	0.054
salicylic acid	0.965	0.016
ferulic acid	0.954	0.024
catechol	0.932	0.002
coniferin	0.897	0.017
spermidine	0.865	0.004
scopolin	0.859	0.023
caffeoylquinic acid	0.849	0.143
coumaric acid	0.832	0.003
caffeic acid	0.768	0.004
lantanilic acid	0.763	0.012
N- vanillylnonanamide	0.749	0.067
camaric acid	0.733	0.005
cinnamic acid	0.706	0.009
synapic acid	0.578	0.003
synapaldehyde	0.528	0.009
oleanolic acid	0.459	0.013

Molecular docking studies: To further refine and validate the screened compounds, docking study was performed to identify interacting residues with the ligands, and to assess binding efficiency. Molecular docking study was carried out by using Molegro Virtual Docker. MVD performs flexible ligand docking, so the optimal geometry of the ligand is determined during the docking. MVD includes MolDock Score and PLANTS Score for evaluating docking solutions. MVD returns multiple poses representing different potential binding modes. Clustering has been used to reduce the number of poses obtained during docking run and the most promising ones were taken. Molecules were prepared for docking; for which bonds, bond orders, explicit hydrogen, charges, flexible torsions, were assigned by the MVD program for both the protein and ligands. The intact protein structure was loaded on to MVD platform for docking process. Ignore distant atoms option is used to ignore atoms far away from the binding site. The search algorithm is taken as Moldock SE and numbers of runs are taken 10 and max iterations were 2000 with population size 50 and with an energy threshold of 100 also at each step least 'min' torsions/translations/rotations are tested and the one giving lowest energy is chosen. Pose clustering was done by tabu based clustering method, using this clustering technique each found solution is added to a 'tabu list': during the docking simulation the poses are compared to the ligands in this 'tabu list'. The Rerank Score uses a weighted combination of the terms used by the MolDock score mixed with a few

addition terms (the Rerank Score includes the Steric (by LJ12-6) terms which are Lennard-Jones approximations to the steric energy – the MolDock score uses a piecewise linear potential to approximate the steric energy). The reranking score function is computationally more expensive than the scoring function used during the docking simulation but it is generally better than the docking score function at determining the best pose among several poses originating from the same ligand. MolDock showed better overall performance in docking simulations when compared with other software.

Docking results for the ligands with each target are shown in Table 6. It is observed that all 18 compounds docked satisfactorily to the potential targets with good (least) docking scores and phytochemicals with least MolDock Score, Rerank Score and greater number of hydrogen bond interactions were selected as promising lead compounds. Out of 144 docked complexes generated, potential hits were selected by MolDock Score, Rerank Score and greater number of hydrogen bond interactions with binding site residues.

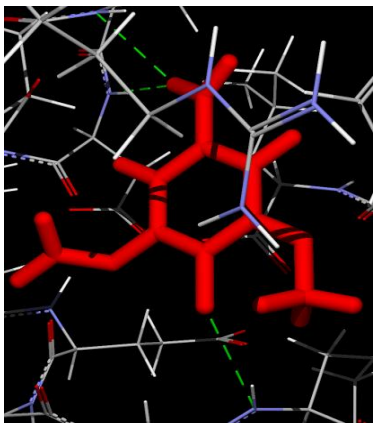


Fig. 1. *The docking poses and interactions of the best docked syringaldehyde- transthyretin complex*

The top hit ligand (syringaldehyde) targeted transthyretin like protein and established three hydrogen bonds with LEU49, CYS51, ASP58 which provide stability to the complex (Fig. 1). From docking results it is evident that the compounds are interacting highly with three targets calreticulin1, GST and transthyretin like protein. Further observed best binding energy scores of the complex were, MolDock score: -183.38, Rerank Score: -103.39 and Ligand efficiency: -6.78, which suggested an energetically favorable interaction of syringaldehyde with transthyretin like protein. The residues involved in hydrogen bonding of docked complexes involved the binding site residues predicted by CASTp server.

Table 6. Binding energy scores and binding residues of phenylpropanoids docked with novel targets of *R. similis*

β-1, 4, endoglucanase					
Ligand	**MolDock Score**	**Rerank Score**	**Ligand efficiency**	**No. of Hbond**	**Binding Residues**
syringaldehyde	-156.23	-136.58	-6.84	9	SER36, SER36, GLY37, THR38, LYS39, LEU40, THR46, ALA47, VAL48
salicylic acid	-149.54	-127.34	0.73	4	Gly37, Ser36, Asn235, Gly237
syringin	-131.64	-112.32	-5.47	9	SER36, SER36, GLY37, THR38, LYS39, LEU40, THR46, ALA47, VAL48
scopolin	-124.28	-89.09	-3.56	6	THR26, TYR30, SER36, GLY37, THR38, PRO457
caffeoylquinic acid	-123.99	-80.16	-3.24	11	GLY37, SER36, GLY37, THR38, LYS39, LEU40, THR46, ALA47, VAL48, HIS51, ASN78
ferulic acid	-123.38	-99.22	-4.94	8	Gly37, Ser36, Asn235, Gly237, Val234, THR38, LYS39, LEU40
catechol	-118.46	-97.25	-4.78	8	Gly37, Ser36, Asn235, Gly237, Val234, THR38, LYS39, LEU40
coniferin	-115.18	-89.65	-3.73	10	Lys39,Thr38, Ala47, Met449, Gly37, Ser36, Asn235, Gly237, Val234, Val234
camaric acid	-115.03	-57.13	-1.39	5	Gly37, Ser36, Asn235, Gly237, Val234
N- vanillylnonanamide	-113.44	-82.53	-3.93	5	GLY37, ASN78, MET212, VAL234, GLY237
lantanilic acid	-110.63	92.71	-4.58	5	Gly37, Ser36, Asn235, Gly237, Val234
coumaric acid	-99.91	-67.66	-5.63	5	Gly37, Ser36, Asn235, Gly237, Val234
caffeic acid	-98.68	-56.85	-4.37	8	SER36, GLY37, THR38, LYS39, LEU40, THR46, ALA47, VAL48
cinnamic acid	-94.23	-65.01	-5.91	3	Gly37, Ser36, Asn235,
synapaldehyde	-93.53	-69.92	-4.65	6	THR26, TYR30, SER36, GLY37, THR38, PRO457
synapic acid	-92.96	-65.01	-4.06	6	THR26, TYR30, SER36, GLY37, THR38, PRO457
spermidine	-80.54	-64.07	-6.44	3	SER36, GLY37, THR38
oleanolic acid	-77.04	-65.36	-0.04	1	GLY37
Calreticulin-1					
Ligand	**MolDock Score**	**Rerank Score**	**Ligand efficiency**	**No. of Hbond**	**Binding Residues**

salicylic acid	-159.64	-113.15	-2.31	3	GLU390, ASP391, ASP391
syringaldehyde	-158.56	-112.96	-5.74	13	ASP389, GLU390, ASP391, GLU392, ASP393, GLU394, GLU396, GLU397, GLU398, LYS399, ASP389, GLU390, ASP391
ferulic acid	-147.79	-96.83	-5.48	4	GLU378, GLU380, ASP391, GLU392
caffeoylquinic acid	-144.01	-103.36	-4.13	12	ASP389, ASP389, GLU390, ASP391, ASP391, GLU392, ASP393, GLU394, GLU396, GLU397, GLU398, LYS399
syringin	-138.13	-107.33	-6.43	12	ASP389, ASP389, GLU390, ASP391, ASP391, GLU392, ASP393, GLU394, GLU396, GLU397, GLU398, LYS399
N- vanillylnonanamide	-136.24	-109.41	-5.21	5	GLU377, GLU378, GLU380, ASP391, GLU392
coniferin	-135.88	-103.28	-4.33	13	ASP389, GLU390, ASP391, GLU392, ASP393, GLU394, GLU396, GLU397, GLU398, LYS399, ASP389, GLU390, ASP391
catechol	-135.87	-98.44	-2.74	6	GLU390, ASP391, ASP391, GLU392, ASP393, GLU394
scopolin	-132.85	-104.18	-4.16	13	ASP389, ASP389, GLU390, ASP391, GLU392, ASP393, GLU394, GLU396, GLU397, GLU398, LYS399, GLU394, GLU396
coumaric acid	-128.62	-98.76	-5.81	5	GLU377, GLU378, GLU380, ASP391, GLU392
spermidine	-120.14	-76.67	-7.66	6	GLU390, ASP391, ASP391, GLU392, ASP393, GLU394
camaric acid	-111.93	-94.03	-2.58	4	GLU390, ASP391, GLU378, GLU380
lantanilic acid	-111.57	-98.33	-2.87	4	GLU390, ASP391, GLU378, GLU380
synapic acid	-103.41	-81.57	-5.09	6	ASP391, ASP391, GLU392, ASP393, GLU394, GLU396
synapaldehyde	-103.11	-77.52	-5.16	4	GLU392, ASP393, GLU394, GLU396
cinnamic acid	-95.63	-86.46	-6.04	2	ASP391, ASP391
caffeic acid	-91.53	-85.38	-5.79	8	GLU377, GLU378, GLU380, ASP391, GLU392, GLU390, ASP391, GLU378,
oleanolic acid	-79.65	-61.94	0.05	2	ASP391, GLU392

Xylanase

Ligand	MolDock Score	Rerank Score	Ligand efficiency	No. of Hbond	Binding Residues
syringaldehyde	-165.59	-133.37	-6.97	16	ILE214, TYR226, SER227, SER228, GLN230, ASN231, LYS232, GLY233, LYS234, VAL236, ALA265, SER266, TYR267, ASN268, THR311, SER266
syringin	-153.92	-120.89	-3.57	10	SER228, GLN230, ASN231, LYS232, GLY233, LYS234, VAL236, ALA265, SER266, TYR267

salicylic acid	-144.38	-86.02	-5.65	2	SER228, SER28
caffeoylquinic acid	-143.34	-106.36	-4.25	16	ILE214, TYR226, SER227, SER228, GLN230, ASN231, LYS232, GLY233, LYS234, VAL236, ALA265, SER266, TYR267, ASN268, THR311, SER266
coniferin	-133.39	-104.91	-4.37	7	SER228, LYS232, GLY233, LYS234, VAL236, ALA265, SER266
scopolin	-131.45	-101.64	-4.06	7	SER227, SER228, ASN231, LYS232, GLY233, LYS234, SER266
ferulic acid	-130.52	-104.07	-5.29	6	SER228, GLN230, ASN231, LYS232, GLY233, LYS234,
catechol	-126.54	-98.07	-5.67	6	SER228, GLN230, ASN231, LYS232, GLY233, LYS234,
N- vanillylnonanamide	-121.07	-91.27	-4.34	4	TYR226, SER227, SER228, GLN230
lantanilic acid	-109.33	-96.31	-0.16	4	ASN231, LYS232, GLY233, LYS234
camaric acid	-105.53	-73.69	1.79	4	ASN231, LYS232, GLY233, LYS234
synapic acid	-102.91	-77.91	-4.86	6	GLN230, ASN231, LYS232, GLY233, LYS234, VAL236
coumaric acid	-102.65	-99.76	-4.98	4	TYR226, SER227, SER228, GLN230
synapaldehyde	-97.96	-71.35	-4.75	6	GLN230, ASN231, LYS232, GLY233, LYS234, VAL236
caffeic acid	-96.98	-84.32	-4.17	8	SER227, SER228, GLN230, ASN231, LYS232, GLY233, LYS234, SER266
cinnamic acid	-84.03	-86.68	-2.42	4	ASN231, LYS232, GLY233, LYS234
spermidine	-83.62	-72.95	-7.29	6	GLN230, ASN231, LYS232, GLY233, LYS234, VAL236
oleanolic acid	-81.25	-62.65	-0.08	6	GLN230, ASN231, LYS232, GLY233, LYS234, VAL236

Cathepsin B-like cysteine proteinase

Ligand	MolDock Score	Rerank Score	Ligand efficiency	No. of Hbond	Binding Residues
syringaldehyde	-149.75	-133.2	-6.54	12	PRO214, PRO214, LEU213, PRO214, HIS215, THR216, MET280, LYS73, LYS73, VAL124, THR127,THR127
syringin	-141.75	-125.93	-3.38	11	PRO214, PRO214, LEU213, PRO214, HIS215, THR216, MET280, LYS73, LYS73, VAL124, THR127
salicylic acid	-139.18	-133.01	-4.15	1	PRO214
caffeoylquinic acid	-136.71	-81.53	-3.26	8	PRO214, PRO214, LEU213, PRO214, HIS215, THR216, MET280, LYS73,
ferulic acid	-136.01	-106.76	-4.34	2	PRO214, LYS73

catechol	-129.01	-110.2	-4.89	2	PRO214, LYS73
coniferin	-128.35	-88.06	-3.66	10	LYS73, VAL124, THR127, ILE128, LEU213, PRO214, HIS215, THR216, MET280, TRP322
scopolin	-127.03	-75.32	-3.08	7	ASN133, SER136, VAL140, ALA143, GLN147, HIS161
N- vanillylnonanamide	-122.32	-97.91	-3.71	5	LEU213, PRO214, HIS215, THR216, MET280
caffeic acid	-112.54	-92.21	-4.78	6	PRO214, LEU213, PRO214, HIS215, THR216, MET280
camaric acid	-107.08	-94.69	-0.35	3	PRO214, HIS215, THR216
coumaric acid	-105.51	-98.01	-4.83	2	PRO214, HIS215
synapic acid	-101.43	-69.52	-4.34	1	PRO214
lantanilic acid	-101.04	-20.26	-0.51	1	LYS73
synapaldehyde	-99.91	-61.94	-4.12	1	LYS73
cinnamic acid	-82.47	-84.65	-4.96	0	
spermidine	-81.43	-74.89	-7.48	5	LEU213, PRO214, HIS215, THR216, MET280
oleanolic acid	-66.76	-51.99	-1.03	1	PRO214

Cathepsin S-like cysteine proteinase

Ligand	MolDock Score	Rerank Score	Ligand efficiency	No. of Hbond	Binding Residues
ferulic acid	-143.18	-153.62	-4.43	6	MET163, PRO226, GLY261, VAL262, MET163, PRO26
catechol	-142.18	-114.38	-4.22	6	MET163, PRO226, GLY261, VAL262, MET163, PRO26
salicylic acid	-133.65	-54.74	-5.47	5	MET163, PRO226, GLY261, VAL262, MET163
N- vanillylnonanamide	-131.17	-103.57	-4.93	4	SER142, LEU143, GLU145, MET163
coumaric acid	-127.93	-113.97	-5.32	2	MET163, PRO226
syringin	-126.54	-115.24	-1.73	7	MET163, PRO226, GLY261, VAL262, MET163, VAL262,GLY261
syringaldehyde	-123.73	-118.24	-8.73	7	MET163, PRO226, GLY261, VAL262, MET163, VAL262,GLY261
caffeoylquinic acid	-121.07	-84.89	-3.39	5	MET163, PRO226, GLY261, VAL262, MET163
coniferin	-117.08	-62.17	-2.59	7	LEU143, GLU145, MET163, PRO226, GLY261, VAL262, MET163
scopolin	-115.88	-63.44	-2.53	6	SER142, LEU143, GLU145, MET163, GLU145, SER142
synapic acid	-99.24	-69.35	-4.33	4	SER142, LEU143, GLU145, MET163

caffeic acid	-98.49	-85.11	-4.74	4	SER142, LEU143, GLU145, MET163
synapaldehyde	-89.71	-68.45	-4.56	2	MET163, PRO226
cinnamic acid	-88.15	-87.26	-6.11	3	MET163, PRO226, PRO226
spermidine	-83.69	-73.75	-7.37	3	MET163, PRO226, PRO226
lantanilic acid	-82.46	-76.14	-1.67	2	MET163, PRO226
camaric acid	-77.31	-79.62	1.94	3	MET163, PRO226, PRO226
oleanolic acid	-33.96	-9.34	0.01	0	

Cytochrome c oxidase subunit III

Ligand	MolDock Score	Rerank Score	Ligand efficiency	No. of Hbond	Binding Residues
syringin	-166.61	-159.68	-3.44	0	
syringaldehyde	-152.61	-123.68	-2.64	0	
salicylic acid	-144.45	-60.79	-6.07	1	SER230
ferulic acid	-142.65	-136.53	-4.75	1	SER230
caffeoylquinic acid	-140.47	-103.03	-4.12	3	PHE240, VAL241, ASP242
catechol	-129.92	-117.39	-2.45	0	
coumaric acid	-122.12	-76.94	-5.57	1	PHE240
N- vanillylnonanamide	-120.19	-112.82	-3.94	3	SER230, TYR231, GLU232
coniferin	-117.85	-72.54	-3.02	2	LEU229, SER230
camaric acid	-114.44	-86.47	-2.11	2	LEU248, TYR249
scopolin	-111.27	-68.97	-2.75	6	LEU229, SER230, TYR231, GLU232, PHE233, SER234
caffeic acid	-108.56	-97.84	-5.21	1	SER230
lantanilic acid	-101.63	-83.49	-2.14	0	
synapic acid	-100.63	-72.58	-4.53	0	
cinnamic acid	-80.66	-89.75	-5.43	0	
synapaldehyde	-79.53	-67.32	-4.48	3	SER230, TYR231, GLU232
spermidine	-74.05	-52.09	-5.24	2	LEU248, TYR249
oleanolic acid	-71.96	-48.95	-1.48	0	

Glutathione S-transferase					
Ligand	**MolDock Score**	**Rerank Score**	**Ligand efficiency**	**No. of Hbond**	**Binding Residues**
syringin	-167.27	-150.45	-3.45	11	TYR176, TYR176,GLY19, GLY19, GLY18, PHE35, GLU36, ASP37, PHE35, GLU36, ASP37
salicylic acid	-155.63	-148.07	-4.68	4	GLY18, GLY19, PHE35, GLU36
syringaldehyde	-151.38	-145.47	-3.63	11	TYR176, TYR176,GLY19, GLY19, GLY18, PHE35, GLU36, ASP37, PHE35, GLU36, ASP37
ferulic acid	-150.48	-114.25	-5.63	5	ASP16, GLY18, GLY19, PHE35, GLU36
caffeoylquinic acid	-140.29	-99.34	-3.97	7	TYR176,GLY19, GLY19, GLY18, PHE35, GLU36, ASP37
lantanilic acid	-135.69	-93.02	-2.38	5	GLY19, ARG25, PHE35, TYR176, TYR208
catechol	-135.17	-99.29	-2.07	4	TYR176,GLY19, GLY19, GLY18
camaric acid	-134.99	-92.78	-2.26	4	GLY19, GLY18, PHE35, GLU36
caffeic acid	-132.06	-97.62	-5.26	5	ASP16, GLY18, GLY19, PHE35, GLU36
scopolin	-124.96	-97.29	-3.89	12	ASP16, GLY18, GLY19, ARG25, PHE35, GLU36, ASP37, TYR38, ARG39, LYS41, ASP46, TYR176
coumaric acid	-124.04	-83.15	-5.26	5	ASP16, GLY18, GLY19, PHE35, GLU36
coniferin	-117.77	-88.35	-3.68	7	TYR176,GLY19, GLY19, GLY18, PHE35, GLU36, ASP37
N- vanillylnonanamide	-115.45	-93.67	-4.46	3	TYR176, PRO211, ALA212
synapic acid	-112.32	-92.64	-4.53	5	ASP16, GLY18, GLY19, PHE35, GLU36
cinnamic acid	-98.43	-66.99	-5.18	2	GLY19, GLY19
oleanolic acid	-94.51	-65.01	-1.97	5	GLY19, GLY18, PHE35, GLU36, ASP37
synapaldehyde	-93.84	-65.87	-4.39	3	ASP37, TYR38, ARG39
spermidine	-90.15	-63.01	-6.63	3	TYR38, ARG39, LYS41

Transthyretin-like protein 3 precursor					
Ligand	**MolDock Score**	**Rerank Score**	**Ligand efficiency**	**No. of Hbond**	**Binding Residues**
syringaldehyde	-188.38	-137.39	-6.78	3	LEU49, CYS51, ASP58
ferulic acid	-183.63	-103.81	-5.62	6	TYR138, ALA140, CYS51, VAL61, PHE87, LEU89

syringin	-178.48	-123.74	-6.71	11	LEU49, CYS51, ASP58, VAL61, PHE149, GLY151, ILE119, ILE121, ILE123, TYR138, ALA140
catechol	-135.63	-128.81	-5.23	4	GLY147, LYS148, PHE149, GLY151
N- vanillylnonanamide	-126.49	-118.43	-3.73	4	GLY147, LYS148, PHE149, GLY151
coumaric acid	-120.97	-84.27	-4.52	3	ASP58, ALA140, CYS51
caffeic acid	-112.46	-97.52	-2.11	8	GLU46, GLY47, LEU49, CYS51, ASP58, VAL61, PHE87, LEU89
caffeoylquinic acid	-109.88	-64.85	-2.59	7	GLU46, GLY47, LEU49, CYS51, ASP58, VAL61, CYS51, LEU89
scopolin	-105.47	-131.43	-1.25	9	VAL45, GLU46, GLY47, LEU49, CYS51, CYS51, ASP58, VAL61, PHE87
salicylic acid	-103.79	-68.05	-4.43	2	CYS51, CYS51
coniferin	-100.37	-93.42	-2.22	7	VAL45, GLU46, GLY47, LEU49, CYS51, CYS51, ASP58
camaric acid	-99.79	-69.42	-1.44	5	CYS51, ASP58, TYR138, ALA140, VAL143
lantanilic acid	-92.82	-83.26	-1.08	3	CYS51, ASP58, VAL61
synapaldehyde	-90.93	-67.99	-4.53	6	ASP58, ALA140, CYS51, CYS51, ASP58, ALA140
synapic acid	-88.55	-55.69	-3.49	2	CYS51, ASP58
spermidine	-85.56	-65.12	-6.51	5	ASP58, ALA140, CYS51, ASP58, ALA140
cinnamic acid	-85.34	-77.53	-4.13	0	
oleanolic acid	-72.53	-62.78	-1.72	3	TYR138, ASP58, VAL143

For further refining the study, a comparative docking study was also performed with a currently used nematicide - carbofuran, PubChem CID 2566 to assess the efficiency of phenylpropanoids over synthetic inhibitor. Carbofuran also interacted highly with the three targets calreticulin1, GST and transthyretin like protein which had high interaction with phenylpropanoids. Phenylpropanoids which have least or similar binding energy scores, ligand efficiency and hydrogen bond interactions were confirmed with docking study. Binding energy scores and binding residues of nematode inhibitor - carbofuran are displayed in Table 7. The study revealed that most of the phenylpropanoids have least doc score than carbofuran (-130.59) and more number of hydrogen bonds. Phytochemicals with least docking score than carbofuran are potential inhibitors of corresponding targets. Based on these, 13 compounds viz. syringaldehyde, ferulic acid, syringin, salicylic acid, caffeoylquinic acid, N- vanillylnonanamide, coniferin, catechol, lantanilic acid, camaric acid, scopolin, caffeic acid and coumaric acid were identified as lead compounds for inhibiting R. similis.

Table 7. Binding energy scores and binding residues of nematode inhibitor- carbofuran with *R. similis* targets

Protein	MolDock Score	Rerank Score	Ligand efficiency	No. of H bond	Binding Residues
Transthyretin-like protein 3 precursor	-130.59	-129.45	-6.02	5	CYS51, CYS51, ASP58
Glutathione S-transferase	-118.19	-70.64	-4.55	4	TYR176,GLY19, GLY19, GLY18
Calreticulin 1	-112.07	-85.04	-4.31	2	GLU392, ASP391
Xylanase	-110.69	-68.57	-4.25	3	LYS232, SER228, SER266
Cytochrome c oxidase subunit III	-103.58	-70.64	-3.98	1	SER230
β-1, 4, *endoglucanase*	-97.26	-6.23	-3.74	1	GLY37
Cathepsin B-like cysteine proteinase	-94.06	-30.81	-3.61	2	PRO214, LYS73,
Cathepsin S-like cysteine proteinase	-93.88	-6.72	-3.61	1	MET163

5. *In vitro bioassay of selected compounds for nematode inhibition*

Nematode inhibition by five phenolic compounds viz. cinnamic acid, coumaric acid, ferulic acid, caffeic acid and N-vanillylnonanamide (NVA) (purchased from Sigma-Aldrich) at four different concentrations were evaluated in an in vitro bioassay. The compounds were dissolved in aqueous DMSO (1%) or distilled water and stock solutions (10 mg ml−1) were prepared and stored at −20◦C after filter sterilization. Compounds were tested for nematode mortality in four concentrations (200, 100, 50, 20 µg ml^{-1}) with three repetitions per concentration in a 96-well plate. Controls were 1% DMSO and distilled water. A sample of

20-25 nematodes, consisting of adults and juveniles of *R. similis*, was taken in 50 µl of water in each well, prior to the addition of the compound. Plates were then incubated for three days and the numbers of alive/dead nematodes were counted at every 24 h under a stereo zoom microscope. Finally, percentage of nematode mortality were calculated. Among the eight compounds, the maximum mortality was observed with the highest concentrations of syringaldehyde (100%), salicylic acid (90), ferulic acid (71%), catechol (71%), coumaric acid (65%), caffeic acid (48%), tannic acid (30%), N-vanillylnonanamide (28%) and cinnamic acid (14%). Mortality of *R. similis* exposed to phenylpropanoids is shown in Table 8 and Fig. 2.

Table 8. Mortality (after 72 h) of *Radopholus similis* exposed to different concentrations of phenyl propanoids.

Compound	**20***	**50**	**100**	**200**
Cinnamic acid	5.7 (13.6)	6.6 (14.9)	9.0 (17.4)	14.3 (22.2)
Coumaric acid	9.6 (18.1)	12.9 (21.1)	38.0 (38.0)	65.0 (53.7)
Ferulic acid	9.5 (18.0)	19.0 (25.8)	37.6 (37.8)	70.9 (57.4)
Caffeic acid	17.7 (24.8)	22.3 (28.2)	30.7 (33.6)	48.0 (43.8)
N-vanillylnonanamide	9.3 (17.8)	11.3 (19.6)	19.3 (26.1)	27.6 (31.7)
CD (5%)	Across columns – 1.8; Across rows – 1.6			
CV%	7.88			

*Figures in parentheses are arc sine transformed values; * µg ml^{-1}*

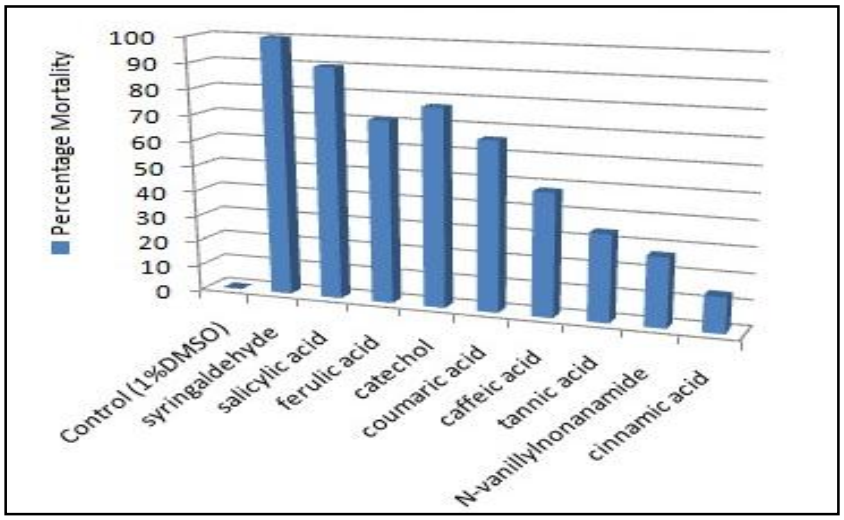


Fig. 2. *Mortality (after 72 hours) of Radopholus similis exposed to selected phenylpropanoids*

6. *In planta bioassay of selected compounds for nematode inhibition*

A greenhouse trial has been laid out with the most promising compound, ferulic acid, using black pepper *R. similis* infested rooted cuttings. Ferulic acid @ 250 and 500 ppm significantly reduced *R. similis* population in black pepper rooted cuttings (Table 9). Besides, the mortality of the nematode infested pepper plants was significantly reduced from 70% in non-treated

plants to 20% in ferulic acid treated plants.

In planta assays with salicylic acid drenching or spraying showed no effect on the R. similis population, even though a progressive reduction in condensed tannins and increase in total phenols was noticed. The experiment will be repeated for confirmation of results.

Table 9. Effect of ferulic acid on Radopholus similis and revival of nematode infested black pepper plants.

Concentration of ferulic acid (in ppm)	Log Nema/g	Height (cm)	Plant mortality (%)
0	3.019	13.77	70
250	2.95	14.52	20
500	2.194	23.12	20

7. *Localization of phenyl propanoids in black pepper roots*

Phenolic cells and lignified walls were visualized in root sections of both HP39 and Sreekara by applying histochemical staining. Phenyl propanoids were localized in roots through staining with diphenylboric acid 2 aminoethyl ester (DPBA) coupled with epifluorescence microscope equipped with a FITC filter (excitation 450–490 nm, suppression LP 520 nm, blue light). Increased presence of phenyl propanoids was observed in the cortical region of HP 39 roots, consequent to nematode inoculation. Lignin was spotted in root sections through two methods viz. Maule staining and Weisner staining. In general, lignification is significantly high in HP 39 roots compared to Sreekara indirectly supporting the inhibition of nematodes by phenyl propanoids in the lignin pathway (Fig. 3). *R. similis* infection increased lignification of endodermis and vascular bundles of both susceptible and resistant black pepper lines and it was greater in cortical cells of Sreekara. Lignification and preformed phenolic cells were not found to be specific features of black pepper lines resistant to nematodes.

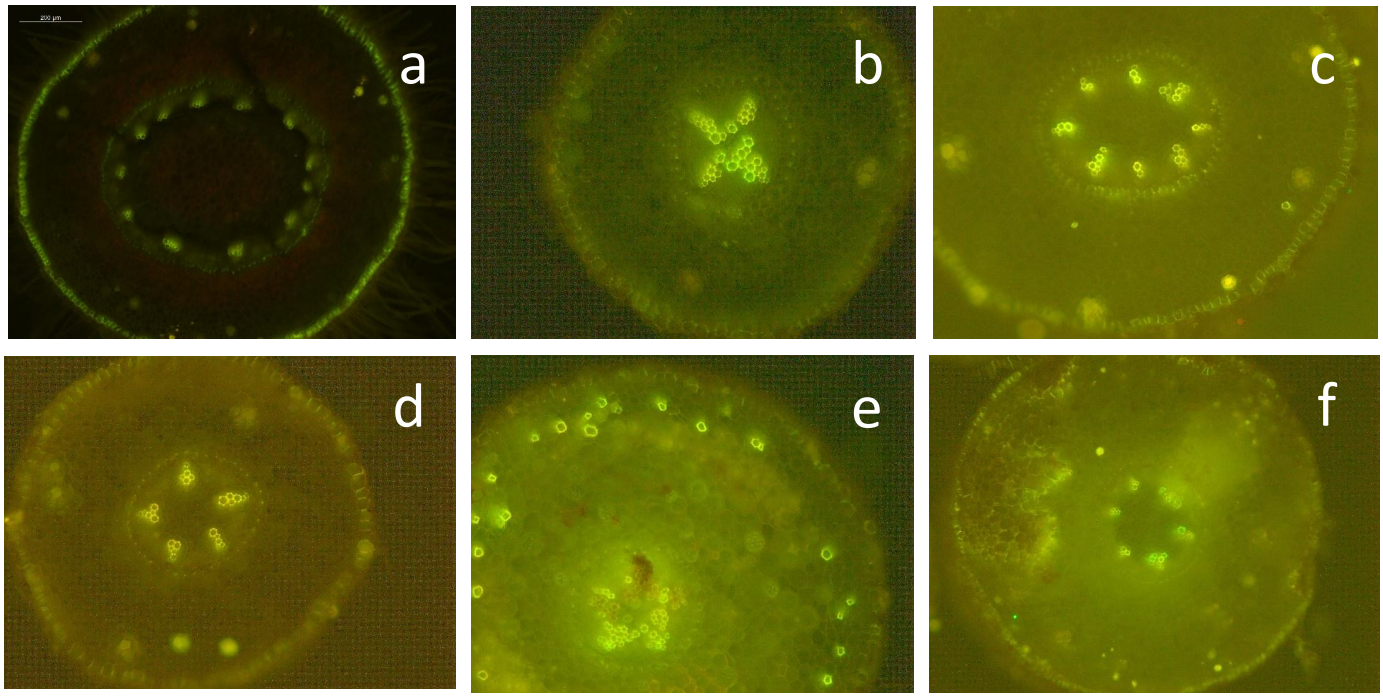


***Fig. 3**: Histochemical changes in black pepper in response to R.similis infection. a-c - HP 39 0, 14 & 21 days and d-f Sreekara 0, 14 & 21 days*

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