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Molecular modeling and docking studies of phytoalexin(s) with pathogenic protein(s) as molecular targets for designing the derivatives with anti-fungal action on *Alternaria* spp. of *Brassica*

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Abstract

The present study used molecular modeling and docking based approaches to test some proteins viz, ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadiene synthase and Glutathione transferase of *Alternaria brassicicola* as possible molecular target of phytoalexins during pathogenesis or defense response. Molecular Operating Environment (MOE) was used to predict 3D structures of above proteins which were subsequently docked with phytoalexins which included Camalexin, Brassilexin, Rutalexin and Spirobrassinin by Molegro Virtual Docker. The results of molecular docking of Spirobrassinin with the above targets showed greater affinity as revealed from binding energy in the range of -73.09 to -94.46 Kcal/mol. Accordingly five derivatives of Spirobrassinin were further designed and docked against each target proteins, so as to detect phytoalexin(s) having the antifungal potential. The molecular modeling and docking experiments identified two derivatives of Spirobrassinins, with binding energy in the range of -77.50 to -85.88 Kcal/mol respectively, which could be used for protection of *Brassica* plants against infection by *Alternaria spp* including *Alternaria brassicicola* and *Alternaria brassicae,* main pathogen of *Alternaria* blight in rapeseed mustard. Further studies and downstream validation would give way to use the above phytoalexin(s) as a substitute for hazardous fungicides to control plant diseases.

Keywords: *Alternaria*, *brassicaceae*, phytoalexin, spirobrassinin, molecular modeling, molecular docking.
Abbreviation: *A. brassicae_Alternaria brassicae;* MOE_Molecular operating environment; PDB_Protein data bank; MVD_Molegro virtual docker; CID_Compound identifier.

Introduction

Brassica is one of the most economically important genus in the *Brassicaceae* family. The world production of *Brassica* has been increasing at a rapid rate in various countries largely with response to the continuing increase in demand for edible oil and its products (Srivastava et al., 2010; 2011). *Brassica* vegetables include cabbage, broccoli, cauliflower, Brussels sprouts, as well as kale, which are consumed all over the world (Podsedek, 2007) and are reported to have both antioxidant and anticarcinogenic properties (Cohen et al., 2000; Chu et al., 2002). *Brassica* crops are heavily challenged by a variety of fungal pathogens and insects, followed by bacterial and viral diseases which have little effect on their yield (Abdel-Farida et al., 2009). The fungal pathogens belonging to *Alternaria* spp which include *Alternaria brassicicola* and *Alternaria brassicae,* cause one of the most economically important diseases of *Brassica* species, above members of the deuteromycetes which cause black spot or leaf spot disease on *Brassica* vegetables. *A. brassicae* can infect virtually several parts of the plant with visible symptoms of infection, which include chlorotic and necrotic lesions on the leaf, inflorescence, petiole, stem, silique and seed (Verma et al., 1994). In addition, the infection can be found in cotyledons at the seedling stage and on the leaves, leaf petiole, stem, inflorescence, siliquae and seeds in adult stage (Kolte et al., 1988). The damaged seeds usually show both internal and external presence of fungus. The yield losses due to this disease vary from 35 to 70% as seen in different species of oilseed *Brassicas* grown in different area of the world. Moreover, oil yield losses due to infected seeds have been reported to range between 15-36% (Ansari et al., 1988). The decline in rapeseed mustard productivity in response to both abiotic and biotic stresses has reported to result from perturbations in cellular networks involved in cell division, cell growth and cell differentiation as investigated in our lab (Pathak et al., 2013; Kumar et al., 2015).

Plants use a complex defense system against pests and pathogens, leading to production of low molecular mass secondary metabolites and compounds with antimicrobial activity, collectively known as phytoalexins (Ahuja et al., 2012). Phytoalexins are a heterogenous group of compounds (Shinbo et al., 2006) that show biological activity towards

variety of pathogens and are considered as molecular markers of disease resistance (Schmelz et a., 2011; Huffaker et al., 2011). The concept of phytoalexin was introduced 70 years ago (Muller et al. 1940) based on the report that potato (*Solanum tuberosum*) tuber tissue infected with an incompatible race of *Phytophthora infestans* develops induced resistance to a compatible race of *P. infestans*. Phytoalexins have been shown to strongly inhibit conidial germination, germ tube elongation and also damage the cell membrane of plant pathogen, whereas the exact mechanism by which phytoalexin exerts its toxicity is still unknown (Sellam et al., 2007). Phytoalexins which are considered essential compounds for plant resistance against pathogens have yet to be characterized, in most species and cultivars (Ahuja et al., 2012). The novel approaches, such as, molecular modeling and docking should open the door for better understanding of the role of phytoalexins in defense against plant pathogens. Better knowledge of the mode of action of phytoalexins and the molecular mechanisms used by plant pathogens to bypass this line of defense should reveal new possibilities for the directed control of phytoalexin production in specific cells and tissues at definite developmental stages. ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadiene synthase and Transferase synthatase are some proteins/enzymes of *Alternaria* spp.; which play a pivotal role in the growth and development of fungus during different stages of pathogenesis of various diseases (Guillemette et al. 2004; Cho et al., 2012; Mamgain et al., 2013; Pochon et al., 2013). The molecular modeling and docking studies can be used to identify important phytoalexins which can neutralize the above proteins during pathogenesis. In this paper, attempts were made to use *in silico* approaches comprehensively for designing of phytoalexin derivatives that can be used for protection of *Brassica* against *Alternaria* spp.

Results and Discussions

Prediction of binding cavity of Alternaria pathogenic proteins

Investigating the binding cavities found in modeled protein structure is a challenging task; many efforts has been made to develop some computational tools that can successfully identify the cavities for scoring and binding affinity prediction with ligand(s) molecule through molecular docking (Wei et al., 2002). The cavity detection algorithm was used dynamically for investigating the cavities by search algorithm guided differential evolution to focus the search during docking simulation. The volumes of cavities present in pathogenic proteins of *Alternaria* were calculated by MVD; Default parameter of MVD was used to predict five cavities in each proteins. Since, the cavity with the largest size and volume is associated with the binding site; therefore, the cavity with the largest volume has been selected as binding site during docking studies (Thomsen et al., 2006; Pathak et al., 2014)

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Docking of phytoalexins with pathogenic proteins of Alternaria

In recent year, the systematic identification of lead compound has gained a lot of attention in agrochemical industries. The progress in Bioinformatics and Computational chemistry facilitated the rapid investigation of agrochemicals for crop plant protection (Avram et al., 2014). Computer aided molecular docking and designing is a rational approach that is often used in agrochemical discovery as an essential tools for screening and optimization of ligands molecules (Lamberth et al., 2013). In 1960s more than 1 kg of agrochemical was usually applied per ha due to lack of knowledge about molecular target, today the use rates can be reduced as 10 g/ha, it is only 10% of that previously required (Schirmer et al., 2012; Lamberth et al., 2013). Therefore, molecular docking of pathogenic proteins including *Alternaria* ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadiene synthase and Glutathione transferase was carried out with each phytoalexin viz., Camalexin, Brassilexin, Rutalexin and Spirobrassinin. Camalexin docked with ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadine synthase and Glutathione transferase with docking energies -77.34, -90.06, -87.95, -88.27, -86.98 and -82.18 kcal/mol respectively. Brassilexin was docked with ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadiene synthase and Glutathione transferase with docking energies -66.45, -73.79, -70.85, -72.74, -68.44 and -68.71 Kcal/mol respectively. Rutalexin was docked with ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadiene synthase and Glutathione transferase with docking energies -67.79, -67.88, -74.32, -72.01, -73.53 and -79.17 Kcal/mol respectively and Spirobrassinin was docked with ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadiene synthase and Glutathione transferase with docking energies -84.00, -82.90, -77.54, -88.45, -94.46 and -73.09 Kcal/mol respectively**.** Hydrogen bonds between phytoalexin(s) and amino acid residues of *Alternaria* pathogenic protein have been depicted in Fig. 3, but no significant interactions were predicted in case of Fusicoccadiene synthase and Glutathione transferase with Camalexin, and ABC transporter with Brassilexin (Fig 3; Table 1).

Identification of agriculturally important lead molecule

The modern agrochemicals interacted with their targets via the same molecular recognition processes, having the potential to inhibit pathogenic protein or activating the defense related pathway for production of antimicrobial compound in crop plants systems could be utilized by plants for protection of their life (Lamberth et al., 2013; Kumar et al., 2015). MVD and its visualizer were used in the study for interaction site analysis and for binding of phytoalexin with *Alternaria* pathogenic protein to find out the residues that are involved in binding (Thomsen et al., 2006). The Spirobrassinin showed highest binding affinity for *Alternaria* pathogenic proteins as revealed from energy value in the range of -73.09 to -94.46 Kcal/mol. Spirobrassinin may be used as agriculturally important lead compounds for protection of *Brassica* which shows H-bond interactions with *Alternaria* ABC transporter ARG700, ASN712 with two hydrogen bond (-84.00) Kcal/mol; PRO48 amino acid residue of Amr1 with one hydrogen bond (-82.90) Kcal/mol; ARG12 amino acid residues of Beta-tubulin with two hydrogen bond (-77.54) Kcal/mol; HIS187, SER40 amino acid residue of Cutinase with two hydrogen bond (-88.45) Kcal/mol; GLN96 amino acid residue of Fusicoccadiene with one hydrogen bond (-94.46) Kcal/mol and GLN4 amino acid residues of Glutathione transferase with one hydrogen bond (-73.09) Kcal/mol (Fig. 3). The hydrogen bonding is very significant in the interaction of biomolecules (Williams et al., 2005). A comparative study with the docking energy values reveals that the phytoalexin Spirobrassinin has better affinity towards the *Alternaria* proteins/enzymes as it has lowest docking energy. This information would prove to be important in designing of spirobrassinin like agriculturally important molecules for protection of *Brassica* (Abdel-Farid et al., 2006).

Table 1. Docking energies of Phytoalexins with Alternaria proteins/enzymes.

S.N.	Alternaria protein/enzymes	Docking energies Kcal/mol	Amino acid residue involved in protein-ligand interactions
Docking energies of Camalexin with Alternaria proteins/enzymes			
1	ABC transporter	-77.34	SER697
2	Amr1	-90.06	PHE3, PHE11
3	Beta-tubulin	-87.95	GLY82, GLY84
4	Cutinase	-88.27	THR184
5	Fusicoccadiene synthase	-86.98	-
6	Glutathione transferase	-82.18	-
Docking energies of Brassilexin with Alternaria proteins/enzymes			
7	ABC transporter	-66.45	-
8	Amr1	-73.79	PHE3, THR12
9	Beta-tubulin	-70.85	VAL14
10	Cutinase	-72.74	SER40
11	Fusicoccadiene synthase	-68.44	SER451, LEU546
12	Glutathione transferase	-68.71	GLY205
Docking energies of Rutalexin with Alternaria proteins/enzymes			
13	ABC transporter	-67.79	ARG700, ASN712
14	Amr1	-67.88	PRO48, ARG61
15	Beta-tubulin	-74.32	THR86
16	Cutinase	-72.01	HIS187
17	Fusicoccadiene synthase	-73.53	CYS600,ASP459
18	Glutathione transferase	-79.17	PHE204, TYR122, GLN211
Docking energies of Spirobrassinin with Alternaria proteins/enzymes			
19	ABC transporter	-84.00	TYR626, ASN712
20	Amr1	-82.90	PRO48
21	Beta-tubulin	-77.54	ARG12
22	Cutinase	-88.45	HIS187, SER40
23	Fusicoccadiene synthase	-94.46	GLN596
24	Glutathione transferase	-73.09	GLN4

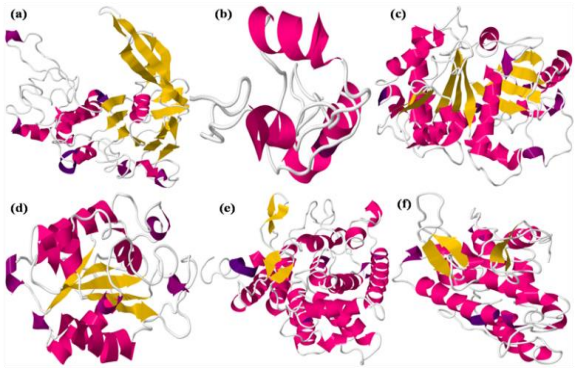

Fig 1. Structure of Alternaria (a) ABC transporter (b) Amr1 (c) Beta-tubulin (d) Cutinase (e) Fusicoccadiene synthase and (f) Glutathione transferase proteins of Alternaria spp.

Designing of the Spirobrassinin derivative

The design and development of scytalone dehydratase inhibitors is one of the most detailed examples that have been reported as fungicides for rice blast disease (Walter, 2002). Nowadays, modern technique like molecular modeling, virtual screening developed primarily in bio-pharmaceutical industries have been fruitfully used in agricultural industries for the discovery and designing of novel agrochemicals (Lamberth et al., 2013).

Molecular docking studies of phytoalexins against pathogenic protein of *Alternaria* revealed that Spirobrassinin could be best phytoalexin for the protection of *Brassica* against infection of Alternaria spp. On the basis of this observation, we designed five derivatives of Spirobrassinin with improved binding affinity towards pathogenic proteins of *Alternaria.* OH and CH_3 functional groups are commonly found in phytoalexins produced by members of the family Fabaceae, Vitaceae, Solanaceae and Poaceae (Ahuja et al., 2012), it may be useful for designing of spirobrassinin derivatives. R position of Spirobassinin structure were chosen for group

Table 2. Docking results of Spirobrassinin derivative's with *Alternaria:* illustrate minimum free energy required for hydrogen bonding and amino acid residues involved in protein-ligand interactions.

S.N.	Alternaria proteins	Spirobrassinin01 R=SOH		Spirobrassinin02 R=OH		Spirobrassinin03 R=CH_3		Spirobrassinin04 R=OCH_3		Spirobrassinin05 R=$SOCH_3$	
		Docking Energy	Interacting AA residues	Docking Energy	Interacting AA residues	Docking Energy	Interacting AA residues	Docking Energy	Interacting AA residues	Docking Energy	Interacting AA residues
1	ABC transporter	-62.35	LEU684, GLU717, SER697	-77.25	VAL696, LEU694, ARG700, SER697	-61.05	SER697, ARG700	-71.38	ASN12, TYR626	-77.50	SER693, ARG700, ASN712
2	Amr1	-62.67	THR12	-74.10	LYS50, PRO48	-63.77	THR44, ASN45	-68.06	--	-56.13	HIS55
3	Beta-tubulin	-62.96	ARG12, GLN84	-68.13	VAL14, VAL16	-63.43	THR86	-74.06	ARG12, GLN84	-72.56	THR86
4	Cutinase	-63.79	ASN83, SER119, TYR118	-84.31	HIS187, PRO185, SER119	-74.52	THR184, SER119	-81.55	THR184, SER119, SER40	-82.75	ASN83, SER119, SER40, TYR118
5	Fusicoccadiene synthase	-69.72	GLN517, ASP455, SER451, SER451	-78.15	CYS600, ARG464, ASP455, ASP459	-65.21	ARG464, ASN587	-76.27	GLN580, ASN587	-85.88	GLN517, SER451
6	Glutathione transferase	-69.72	SER59, SER78	-76.32	GLN211, GLN4, ASN3	-70.13	SER78	-72.83	SER78	-77.51	SER78

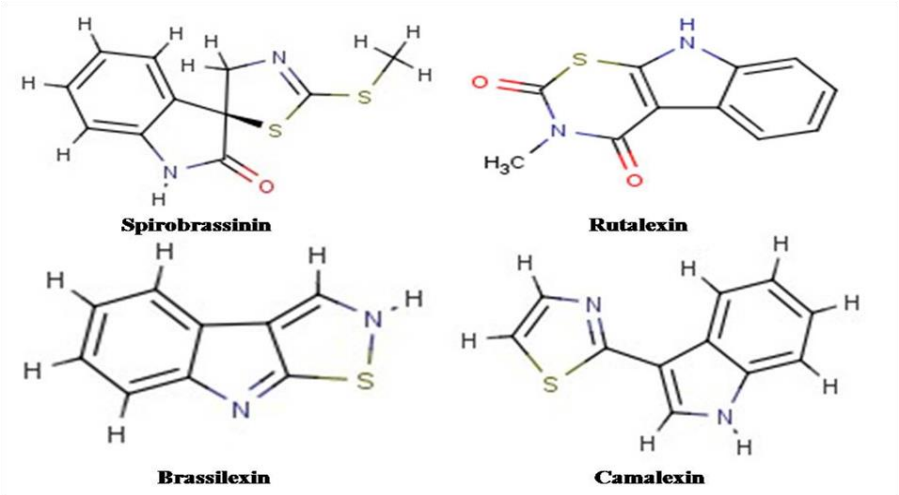

Fig 2. Structures of selected phytoalexins produced by members of the *Brassicaceae.*

Table 3. Physicochemical properties (Chemical formula, Molecular weight, LogP, H-bond donar and acceptors, Polar surface area in (2D), Polarizability, Van der Waals surface Area in (3D), pI and Refractivity) of each selected phytoalexins.

S.N	Properties	Camalexin	Brassilexin	Spirobrassinin	Rutalexin
1	Chemical formula	$C_{11}H_8N_2S$	$C_9H_6N_2S$	$C_{11}H_{10}N_2OS_2$	$C_{11}H_{10}N_2O_2S$
2	Molecular weight (g/mol)	200.25962	174.22234	250.3399	234.274
3	LogP	2.76	2.60	2.76	1.41
4	H-Bond donar	1	1	1	1
5	H-Bond acceptor	2	2	4	3
6	Polar Surface Area (2D) (Å)	28.68	28.68	41.46	49.41
7	Polarizability	23.64	20.18	26.18	23.63
8	Van der Waals Surface Area (3D) ($Å^2$)	254.42	208.28	302.40	286.34
9	pI	8.14	6.40	7.85	8.48
10	Refractivity	67.45	58.41	69.39	62.86

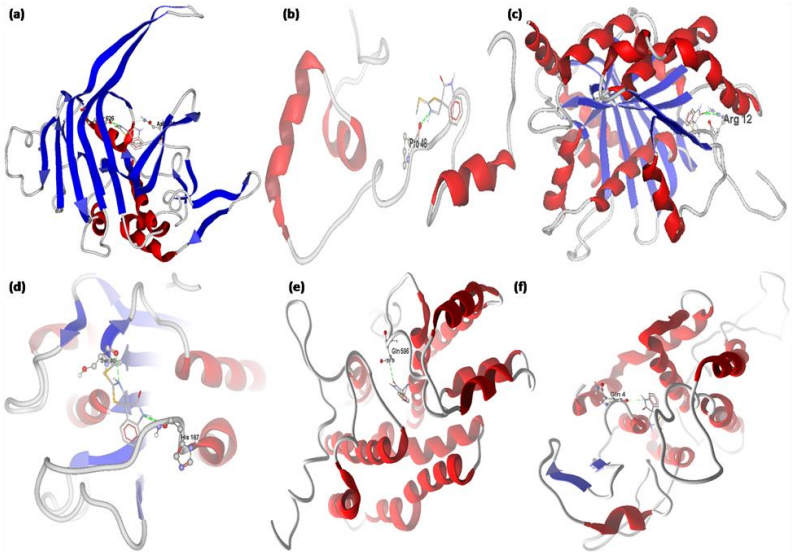

Fig 3. Docked structure of Spirobrassinin with (a) ABC transporter (b) Amr1 (c) Beta-tubulin (d) Cutinase (e) Fusicoccadiene synthase and (f) Glutathione transferase proteins of *Alternaria* spp.

Table 4. Physiochemical properties (Chemical formula, Molecular weight, LogP, H-bond donar and acceptors, Polar surface area in (2D), Polarizability, Van der Waals surface Area in (3D), pI and Refractivity) of each designed derivatives of Spirobrassinin.

S.N.	Properties	Spirobrassinin01	Spirobrassinin02	Spirobrassinin03	Spirobrassinin04	Spirobrassinin05
1	Chemical formula	$C_{10}H_8N_2O_2S_2$	$C_{10}H_8N_2O_2S$	$C_{11}H_{10}N_2OS$	$C_{11}H_{10}N_2O_2S$	$C_{11}H_{10}N_2O_2S_2$
2	Molecular weight (g/mol)	252.313	220.248	218.275	234.274	266.339
3	LogP	2.22	1.86	1.38	1.97	2.60
4	H-Bond donar	2	2	1	1	1
5	H-Bond acceptor	3	3	2	3	3
6	Polar Surface Area (2D) (Å)	61.69	61.69	41.46	50.69	50.69
7	Polarizability	25.01	21.90	23.09	23.81	26.91
8	Van der Waals Surface Area (3D) ($Å^2$)	280.51	257.75	277.86	50.69	317.68
9	pI	6.73	7.64	8.43	7.37	6.94
10	Refractivity	66.40	58.61	61.47	63.36	70.88

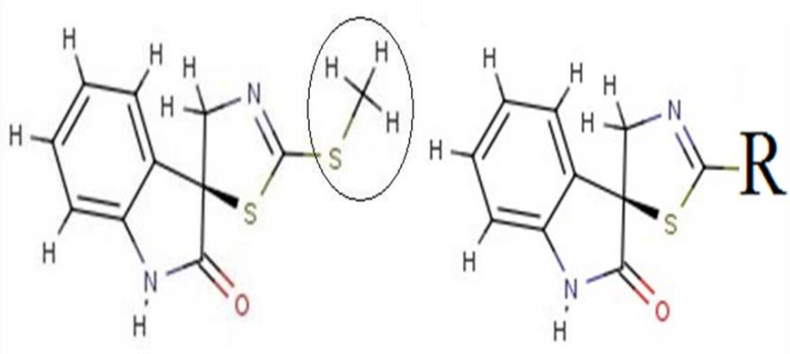

Fig 4. Chemical structure of Spirobrassinin: Modification point for designing of derivatives Spirobrassion01 to Spirobrassinin05 by substituting SCH_3 group was shown by R.

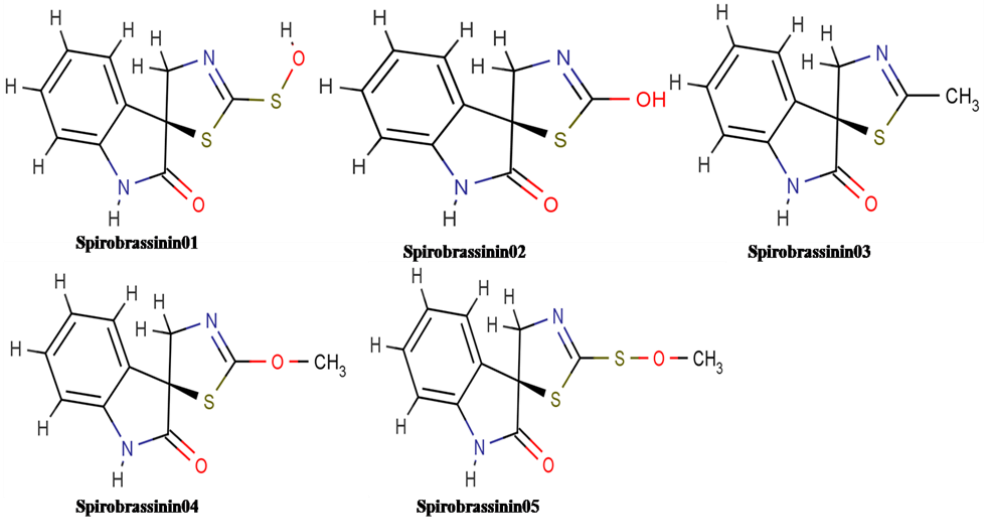

Fig 5. Designed derivatives of Spirobrassinin ((3S)-2'-methylsulfanylspiro[1H-indole-3,5'-4H-1,3-thiazole]-2-one).

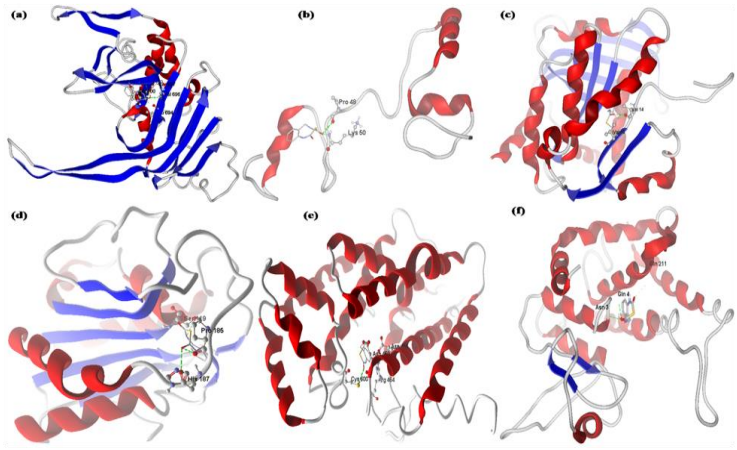

Fig 6. Docked structure of Spirobrassinin02 with (a) ABC transporter (b) Amr1 (c) Beta-tubulin (d) Cutinase (e) Fusicoccadiene synthase (f) Glutathione transferase proteins of *Alternaria* spp.

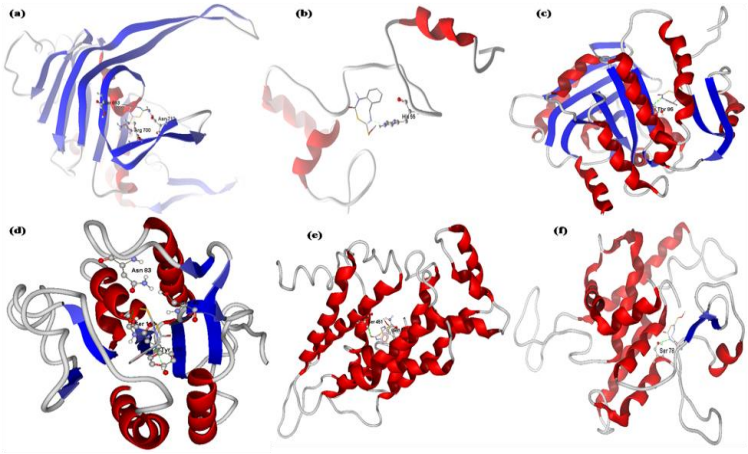

Fig 7. Docked structure of Spirobrassinin05 with (a) ABC transporter (b) Amr1 (c) Beta-tubulin (d) Cutinase (e) Fusicoccadiene synthase (f) Glutathione transferase proteins of *Alternaria* spp.

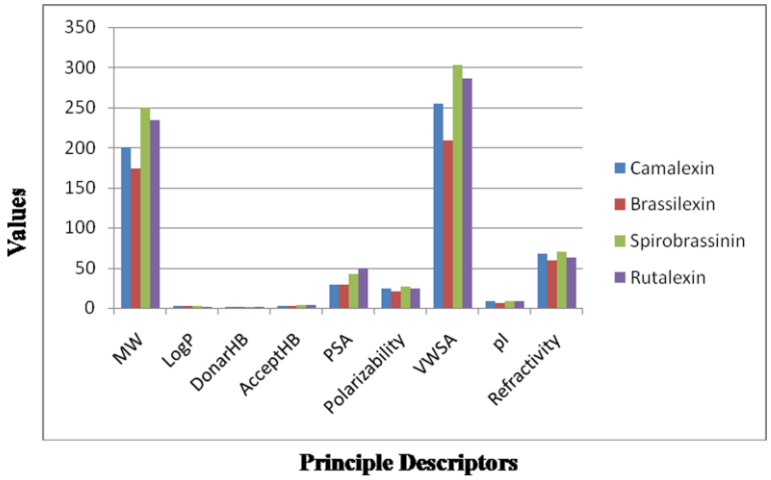

Fig 8. Values of principal descriptors for phytoalexins.

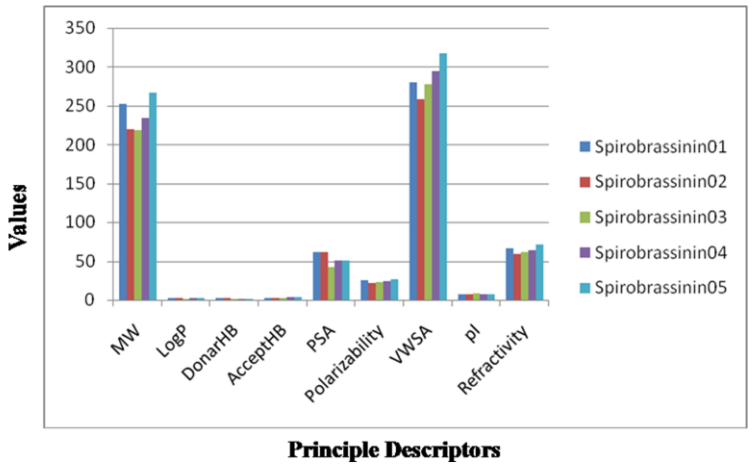

Fig 9. Values of principal descriptors for Spirobrassinin derivatives

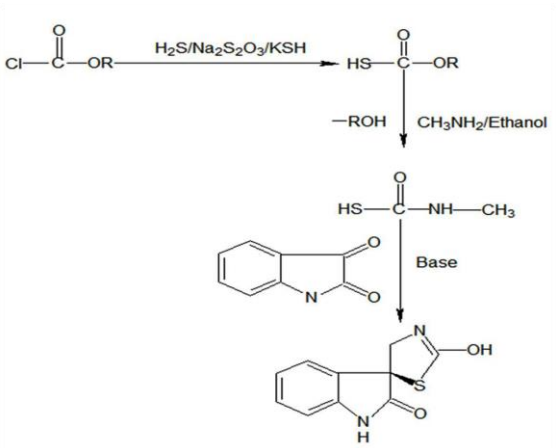

Fig 10. Preparation of Spirobrassinin02 **[**(3S)-5'-hydroxy-1,2-dihydro-3'H-spiro[indole-3,2'-[1,4]thiazole]-2-one].

replacement (SCH_3 by OH and CH_3) to order design derivatives with improved affinity towards pathogenic protein of *Alternaria* than Spirobrassinin, although Spirobrassinin already has good affinity to pathogenic protein of *Alternaria* among all phytoalexins, taken for study (Fig. 2). No changes have been made in other part of Spirobrassisin, because these parts of Spirobrassinin were involved in protein-ligand interactions which are considered as pharmacophore, and these parmacophoric parts were responsible for inhibition of pathogenic proteins. (Fig. 4, 5).

Molecular docking studies of Spirobrassinin derivative with pathogenic proteins of Alternaria

The phytoalexins produced during pathogenesis of *Alternaria brassicicola* was investigated and it was found out that Spirobrassinin is the major phytoalexin produced in infected leaves of *Brassica juncea* (Pedras *et al.*, 2009). The present studies have identified that Spirobrassinin is useful for the inhibition of *Alternaria* pathogenic protein. Therefore, Molecular docking of Spirobrassinin derivatives with *Alternaria* ABC transporter, Amr1, Beta-tubulin, Cutinase, Fusicoccadiene synthase and Glutathione transferase was carried out. Spirobrassinin01 was found to interact with *Alternaria* proteins with energy value in the range of -62.35 to -69.76 Kcal\mol; other derivatives of Spirobrassinin (Spirobrassinin02- Spirobrassinin05) interacted with same proteins of *Alternaria* with energy value in the range of -68.13 to -84.31, -61.05 to -74.52, -68.06 to -81.55 and -56.13 to -85.88 Kcal/mol respectively (Table 2). The results of the present studies identified compound Spirobrassinin02 and Spirobrassinin05 with improved affinity towards the pathogenic proteins of *Alternaria* as compared to Spirobrassinin.

Analysis of Protein-ligand interactions and physicochemical properties of Spirobrassinin

Phytoalexins accumulation in the *Brassicas* after exposure to *Alternaria* and their role in disease resistance have been examined by many researchers (Saharan et al., 2015). The results of the present study have shown that Spirobrassinin01 binds *Alternaria* ABC transporter LEU684, GLU717 and SER697 with four hydrogen bonds; Amr1 THR12 with two hydrogen bonds; Beta-tubulin ARG12 and GLN84 with three hydrogen bonds; Cutinase ASN83, SER119, TYR118 with three hydrogen bonds; Fusicoccadiene synthase GLN517, ASP455 and SER451 with four hydrogen bonds and Glutathione transferase SER59, SER78 with two hydrogen bonds. Spirobrassinin02 binds *Alternaria* ABC transporter VAL696, LEU694, ARG700 and SER697 with seven hydrogen bonds; Amr1 LYS50, PRO48 with three hydrogen bonds; Beta-tubulin VAL14,VAL16 with two hydrogen bonds; Cutinase HIS187, PRO185 and SER119 with three hydrogen bonds; Fusicoccadiene synthase CYS600, ARG464, ASP455, ASP459 with four hydrogen bonds and

Glutathione transferase GLN211, GLN4, ASN3 with three hydrogen bonds (Fig. 6). Spirobrassinin03 binds *Alternaria* ABC transporter SER697, ARG700 with two hydrogen bonds, Amr1 THR44, THR45 with three hydrogen bonds, Beta-tubulin THR86 with one hydrogen bonds, Cutinase THR184, SER119 with three hydrogen bonds, Fusicoccadiene synthase ARG464, ASN587 with two hydrogen bonds and Glutathione transferase SER78 with two hydrogen bonds. Spirobrassinin04 binds at *Alternaria* ABC transporter ASN12, TYR626 with three hydrogen bonds, Beta-tubulin ARG12, GLN84 with three hydrogen bonds, Cutinase THR184, SER119 and SER48 with four hydrogen bonds, Fusicoccadiene synthase GLN580, ASN587 with two hydrogen bonds and Glutathione transferase SER78 with two hydrogen bonds interactions. There were no significant hydrogen bonding found in between Amr1 with Spirobrassinin04. Spirobrassinin05 binds at *Alternaria* ABC transporter SER693, ARG700, ASN712 with four hydrogen bonds, Amr1 HIS55 with one hydrogen bonds, Beta-tubulin THR86 with one hydrogen bonds, Cutinase ASN83, SER119, SE40 and TYR118 with four hydrogen bonds, Fusicoccadiene synthase GLN517, SER451 with two hydrogen bonds and Glutathione transferase SER78 with two hydrogen bonds (Fig. 7). Finally, the comparison between protein-ligand interactions of each Spirobrassinin derivative with pathogenic protein(s) of *Alternaria* suggested that the Spirobrassin02 and Spirobrassin05 might be more effective.

The physicochemical properties of phytoalexins and designed derivative of Spirobrassinin were predicted by MarvinSketch to evaluate the drug likeness. The following 9 principal descriptors were included in the study: molecular weight (MW), LogP, H-Bond donar (DonarHB), H-Bond acceptor (AcceptHB), Polar Surface Area 2D (PSA), Polarizability, Van der Waals Surface Area 3D (VWSA), pI and Refractivity (Fig. 8, 9). According to Lipinski's rule of five a drug will illustrate good ADME (absorption, distribution, metabolism and excretion) properties if it's logP value is less than 5, Hydrogen bond donor should be less than 5, Hydrogen bond acceptor should be less than 10 and Molecular weight should be less than 500 (Lipinski et al., 2001). In order to have good cell membrane permeability a molecule should have polar surface area (PSA) less than 140 Å. As phytoalexins and designed derivative of spirobrassinin possessed PSA value less than 140 Å, it was predicted that they have good cell membrane permeability. Whereas Molar refractivity between 40-130 is an indication of better molecules (Table 3 and Table 4). The Spirobrassinin02 and Spirobrassinin05 having molecular weight 220.248 and 266.339, LogP 1.86 and 2.60, H-bond donar 2 and 1, H-bond acceptor 3 and 3, Polar surface area 61.69 and 50.69, Polarizability 21.90 and 26.91, Van der waals surface area 257.75 and 317.68, pI 7.64 and 6.94 as well as Refractivity 58.61 and 70.88 respectively (Table 4) and have shown drug confirmed behavior which might play a vital role in prevention and management of agriculturally important diseases in crops (Walter, 2002).

Prospects of Spirobrassinin as the agriculturally important molecules for protection of Brassica spp.

Phytoalexins plays an important role in plant resistance against plant pathogens, not only in dicot species but also in monocots (Schmelz et al., 2011; Ahuja et al., 2012). It has recently been shown that attack of maize stem by *Rhizopus microspores* and *Collectotrichum graminicola* induces the accumulation of six ent-kauranne-related diterpenoids, collectively termed kauralexins which inhibit the growth of these pathogens (Schmelz et al., 2011).

The results of present study clearly revealed that phytoalexin spirobrassinin, could act as a lead molecule for the prevention of fungal diseases. Spirobrassinin and its derivatives are small hydrophobic molecules that could cross cell membranes due to ideal logP value and low molecular weight. This should support diffusion of this hydrophobic molecule through the membrane. We have found that designed derivatives of sprirobrassinin viz., spirobrassinin02 and spirobrassinin05 showed highest affinity towards pathogenic proteins of *Alternaria* but spirobrassinin05 is unstable due to S-O-CH_3 linkage. Therefore, Spirobassinin02 may be useful for protection of *Brassica* spp against fungal diseases including *Alternaria* blight.

Synthetic route for Spirobrassinin02

Synthetic chemistry provides a unique opportunity for the synthesis and development of agriculturally important molecules that enhance plant performance and secure yield potential (Lamberth et al., 2013). Advances in recent technology will need to develop novel molecules having potential to protect life of crop plants that will be ultimately increasing agricultural productivity (Walter, 2002; Liu et al., 2014). We have developed a possible synthetic route for the synthesis of spirobrassinin02 and related derivatives as antifungal molecule for protection of *Brassica* against *Alternaria spp*. Spirobrassinin02 may be synthesized by the following methods (Fig.10).

Materials and Methods

Sequence retrieval

The FASTA sequences of the target pathogenic proteins of *Alternaria brassicicola* (1-Entry name: Q2XNF3; Protein name: ABC transporter; Length: 1500, 2-Entry name: G3F820; Protein name Amr1; Length: 1030, 3-Entry name: O74656; Protein name: Beta-tubulin; Length: 337, 4- Entry name: P41744; Protein name: Cutinase; Length: 209, 5-Entry name: C9K2Q3; Protein name: Fusicoccadiene synthase; Length: 697, 6-Entry name: QS2PH5; Protein name: Glutathione transferase; Length: 259) were obtained from UniProt database (http://www.uniprot.org).

Target structure modeling and validation

The three dimensional structures of *Alternaria* proteins/enzymes were made by using homology modeling algorithm with the help of MOE (Molecular Operating Environment) (http://www.chemcomp.com/) (Fig. 1). In order to construct the structure of each protein a template for homology modeling was searched with PDB search Program of MOE. The final structures were determined after constructing and evaluating 3D models. Structural refinements through energy minimization were performed using energy minimization tool keeping parameter value constant for all structures. The minimized structures were finally saved as pdb file format (Labut, 2008a, b; Labute, 2010; Feldman et al., 2010; Almagro et al., 2011).

Retrieval and preparation of ligand molecules

The structure of phytoalexins derived from Brassicaceae family viz., Camalexin (CID: 636970), Brassilexin (CID: 189690), Spirobrasinin (CID: 188830) were retrieved from

Pubchem database of NCBI (National Centre for Biotechnology Information) (http://pubchem.ncbi.nlm.nih.gov) and the structure of Rutalexin was drawn by MarvinSkretch (http://www.chemaxon.com/products/marvin/marvinsketch/) software. The three dimensional coordinates of ligand molecules were generated by MarvinSketch and saved in pdb file format for docking studies (Fig. 2).

Molecular docking approach

Molegro Virtual Docker (MVD) was used for docking studies. It requires a 3D structure of both protein and ligand and performs flexible ligand docking, so the optimal geometry of the ligand is determined during docking (Thomsen et al., 2006). MVD MolDock Score uses algorithm based of the Differential Evolution (DE) algorithm; the MolDock Score energy, E_{score}, is defined by Equation (I), where E_{inter} is the ligand-protein interaction energy and E_{intra} is the internal energy of the ligand. E_{inter} is calculated according to Equation (II).

$$E_{score} = E_{inter} + E_{intra} \quad \text{(I)}$$

$$\left(E_{inter} = \sum_{\text{i ligand}} \sum_{\text{j ligand}} E_{PLP}(r_{ij}) + 332.0 \frac{q_i q_j}{4r^2_{ij}} \right) \quad \text{(II)}$$

The term E_{PLP} is a "piecewise linear potential" using two different parameters, one for the approximation of the steric term such as vander Waals between atoms and another for the potential for hydrogen bonds (Yang and Chen, 2004); it depicts the electrostatic interactions between charged atoms.

$$\left(E_{intra} = \sum_{\text{i ligand}} \sum_{\text{j protein}} E_{PLP}(r_{ij}) + \sum_{\text{Flexible bond}} A\,[1-\cos(m\theta-\theta)] + E_{clash} \right) \quad \text{(III)}$$

E_{intra} is defined by Equation (III). The first term in Equation (III) calculates the total energies involving pairs of atoms of the ligand, except those connected by two bonds. The second term stands for the torsional energy, where θ is the torsional angle of the bond. The average of the torsional energy bond contributions was used if several torsions have to be determined. The word E_{clash} defines a penalty of 1 000 kcal/mol if the distance between two heavy atoms (more than two bonds apart) is smaller than 2.0 Å, ignoring infeasible ligand conformations. The candidates with the best conformational and energetic results were selected (Thomsen et al., 2006).

Conclusion

The present *in silico* studies provides an insight about the interaction of phytoalexins with pathogenic protein(s) of *Alternaria* to explain the mode of inhibition of fungal activity. Our results demonstrated that the docking of phytoalexin with pathogenic protein of *Alternaria*, suggested that phytoalexin spirobrassinin is a lead molecule against *Alternaria.* Based on this data, appropriate modification in the structure of spirobrassinin has been done to design potential agriculturally important molecule(s) for protection of *Brassica* spp. Further studies regarding protein-ligand interaction would pave the way to use phytoalexins as a substitute for presently used synthetic fungicides that cause damage to the environment. Wet lab experimentation is needed to confirm its efficacy and potency of such phytoalexin derivatives having anti-fungal potential for curtailing the incidence of *Alternaria* blight disease of *Brassica*.

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