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Inhibition of rice-blast fungus *Magnaporthe oryzae* by *Piper betle* extracts: *in vitro* evidence and *in silico* prediction

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ABSTRACT

Developing new antimicrobial agents towards *Magnaporthe oryzae* based on *Piper betle* extracts is practicable if an inhibition mechanism is known. The information for the retrieval was collected from experimental investigations and computational researches on the inhibitability of the plant extract compositions (**P1 – P14**) towards the fungus trehalose-6-phosphate synthase (**PDB-6JBR**). Gas chromatography-mass spectrometry characterisation determines 4-Chromanol (**P5**), 1'-Hydroxychavicol acetate (**P6**), Eugenol acetate (**P7**), and 4-Allyl-1,2-diacetoxybenzene (**P8**) making up the majority of *Piper betle* extract composition. Bio-assays provide experimental evidence of a total antifungal effect towards *M. oryzae*. Docking-based simulation confirms the significant static stability of **P5-6JBR**, **P6-6JBR**, **P7-6JBR**, and **P8-6JBR**. QSARIS analysis exceptionalises bio-compatibility of **P5**, **P6**, **P7**, and **P8**. The results prove the antifungal potentiality of *Piper betle* extracts and suggest trehalose-6-phosphate synthase as a promising target for *M. oryzae* inhibition.

Introduction

Piper betle Linn. (Piperaceae) commonly known as betel vine is a popular medicinal plant in Asia.[1] *Piper betle* leaves have been used as a traditional medicine to treat a variety of health conditions thanks to their antibacterial, antifungal and antioxidant properties.[2] Traditionally, betel leaves are used for vaginal douching,[3] as a gargle mouthwash, and as a treatment for dental problems, headaches, arthritis, and joint pain .[4] The betel leaf juice is also used to treat skin ailments.[5] *Piper betle* leaves consist of many chemical components such as betal-phenol, chavicol and other phenolic compounds that are known to have strong potentials in anti-fungal, anti-bacterial properties.[6] Some studies have shown that *Piper betle* leaves perform high efficiency on bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, etc.[7] In fact, the fungicidal effects of *Piper betle* extracts against various fungal species including *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus parasiticus*, *C. albicans*, *Candida glabrata*, *Candida krusei*, *Candida neoformans*, *Candida parapsilosis*,

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Candida tropicalis, *Epidermophyton floccosum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum canis*, and *Microsporum gypseum* were also reported [7].

Rice blast disease caused by an ascomycete fungus *Magnaporthe oryzae (M. oryzae)* is one of the most destructive rice diseases in the world and able to cause a yield loss of up to 30 % [3]. The disease attacks the leaves, culms, branches of the panicle and the floral structures.[8] To prevent plant diseases, the main method is still using industrial pesticides. Inappropriate overusing these chemical drugs has made the pest capable of forming resistance. Moreover, this also causes serious environmental pollutions and intoxication to residues of plant in agricultural products, inevitably affecting the human health and life .[9, 10] Therefore, it is necessary to develop new safe and effective antimicrobial agents that could be applied in agricultural fields. *Piper betle* leaves which highly abundant and inexpensive, could be an effective alternative solution.

The cellular type *M. oryzae*, a heterothallic fungus, infects the plant by its conidia via appressoria, accumulates a high concentration of glycerol, and forms a dense layer of melanin. The accumulation of the melanin is an essential step before the appressoria helps *M. oryzae* penetrate into host plants. The synthesis of melanin is aggressively catalysed by glycosyltransferase superfamily, especially trehalose-6-phosphate synthase (PDB-6JBR). The structure of *M. oryzae* protein 6JBR is well-determined (https:// 10.2210/pdb6JBR/pdb) and given in Figure 1 [11]. Therefore, a bio-inhibition of *M. oryzae* might highly relate to this enzyme.

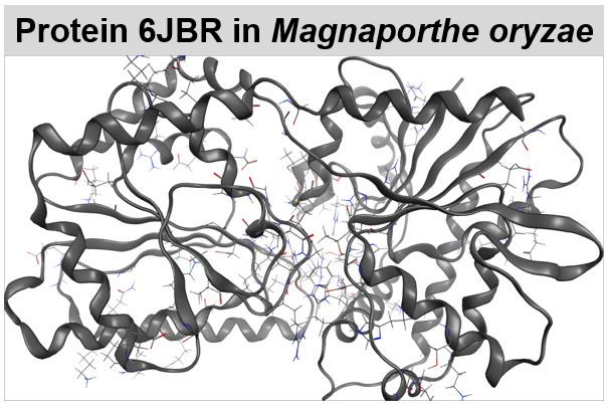

Figure 1: Crystal structure of protein 6JBR in *Magnaporthe oryzae*.

In this study, the chemical compositions of the ethanol extract of *Piper betle* leaves in Thua Thien Hue province in Vietnam were determined and their inhibitability towards fungus *Magnaporthe oryzae* was investigated based on both *in vitro* and *in silico* conditions. The former is for collecting experimental evidence, and the latter is to provide a comprehensible mechanism.

Material and methods

Experimental method

Materials

Fresh *Piper betle* leaves were collected in Phong Dien district, Thua Thien Hue province in March, 2020 and identified at the Department of Biology, University of Sciences, Hue University.

Magnaporthe oryzae was isolated from rice samples in Thua Thien Hue province that had been infected by blast diseases following a standard procedure from IRRI (International Rice Research Institute, 1997) [11] and preserved in Plant Disease Research Laboratory, Plant Protection Department, Hue University of Agriculture and Forestry, Hue University (Hue City, Viet Nam).

Characterisation of Piper betle extract

The *Piper betle* leaves were picked, washed by water, dried at a temperature of 50 ± 2 ºC, and grounded into powder (1-2 mm in size). The *Piper betle* powder was under an extraction using 70 % ethanol by a maceration method for three times, 24 hours each at ambient temperature. The ethanol solvent was removed by a rotary evaporator at 60 °C to obtain a crude extract of *Piper betle*.[2] Chemical constituents of the *Piper betle* extract were determined by a gas chromatography-mass spectrometry (GC-MS).

Antifungal assay on Magnaporthe oryzae

Potato dextrose agar (PDA) was prepared and cooled to 50 °C. *Piper betle* extract was dissolved in 10 % dimethyl sulfoxide (DMSO) by different concentrations (i.e. 0.2; 0.4; 0.6 and 0.8 %). The media were then transferred into petri dishes (9 cm in diameter) with three dishes for each concentration. Mycelial discs (6 mm in diameter) of *Magnaporthe oryzae* were cut and put into the middle of the petri dishes. The diameters of the colonies were determined every day. *M. oryzae* inhibition was measured by the formula: I = [(C-T)/C]x100 %,[12] with *I* is *M. oryzae* inhibition; *T* and *C*

are mycelial disc diameter of the treatment and control, respectively [13].

Computational method

Molecular docking simulation

Molecular docking simulation was implemented on MOE 2015.10 software. A typical procedure follows three steps [14–17].

a) Pre-docking preparation: Structural data of protein 6JBR (DOI: 0.2210/pdb6JBR/pdb) were downloaded from Worldwide Protein Data Bank. The proteins and their 3D protonation were prepared by MOE QuickPrep function. Determination of protein active zones based upon a radius set at 4.5 Å from their amino acids and the inhibitory ligands. The preparatory protein structures obtained were saved in format *.pdb for docking simulation. Independently, the compounds were structurally optimised by Conj Grad.

b) Docking investigation and post-docking analysis: Simulation on intermolecular interaction between the investigated agents was performed on MOE 2015.10 and the obtained inhibitory structures were saved in format *.sdf.

c) Post-docking analysis: The inhibitability of a certain duo-system was primarily predicted by docking score (DS) energy. Intermolecular interactions formed between the ligands and in-pose amino acids of the proteins were also probed. These include hydrophilic binding and hydrophobic interaction. The complex static conformation was evaluated by a value of root-mean-square deviation (RMSD). Ligand conformation and orientation in its inhibited-protein active site was visualised.

QSARIS analysis

Physicochemical compatibility of *Piper betle* composition was evaluated by molecular mass (Da), polarizability ($Å^3$) and volume or size (Å), and dispersion coefficients (logP and logS) obtained from QSARIS system with Gasteiger–Marsili method.[18] Lipinski's rule of five was used as a reference, a well-known set of indicators to predict drug-likeness.[19] According to Lipinski's criteria, a well membrane-permeable molecule should satisfy the requirements: *(1)* Molecular mass < 500 Da; *(2)* no more than 5 groups for hydrogen bonds; *(3)* no more than 10 groups receiving hydrogen bonds; *(4)* the value of logP is less than +5 (logP < 5).[20,21]

Results and discussion

Experiment

Chemical composition

The presence of active compounds and their percentage composition in ethanol extract from leaves of *Piper betle* determined by GC-MS analysis are presented in Table 1. There are fourteen compounds in the ethanol extract of *Piper betle* leaves were identified, accounting for 93.45 % of the composition.

The main constituents in the leaves of *Piper betle* were 4-Chromanol (49.90 %), 1'-Hydroxychavicol acetate (13.23 %), 4-Allyl-1,2-diacetoxybenzene (11.77 %), Eugenol (7.88 %), Eugenol acetate (3.45 %) and β-Sitosterol (2.49 %). Therefore, the overall bio-activity of *Piper betle* extracts are preliminarily considered likely related to these compounds.

Antifungal properties

Concentrations of *Piper betle* extract used for antifungal effect test were 0.0, 0.2; 0.4, 0.6 and 0.8 %. Observation on *M. oryzae* inhibition was recorded after 3, 5 and 7 days of incubation, which are shown in Figure 2 and summarised in Table 2. Overall, by the increase of the concentration of *Piper betle* extract, the mycelial disc diameters decrease, thus better is the *M. oryzae* inhibition.

In particular, with an increase of *Piper betle* extract concentrations from 0.4 to 0.8 %, the inhibiting effect to the fungus reaches 100 %, i.e. complete inhibition to the growth of *M. oryzae*. This means the *Piper betle* extract concentration of 0.4 % was the minimum inhibition concentration (MIC). In summary, the experimental evidence reveals a significant in-reality inhibition effect of *Piper betle* extract towards *M. oryzae*, although the mechanism is yet unclear.

Table 1: Identification of major bioactive compounds in Piper betle extract

Symbol	Substance	Formula	Percentage (%)
P1	Chavicol	$C_9H_{10}O$	0.48
P2	Eugenol	$C_{10}H_{12}O_2$	7.88
P3	β-Caryophyllene	$C_{15}H_{24}$	1.22
P4	α-Caryophyllene	$C_{15}H_{24}$	0.19
P5	4-Chromanol	$C_9H_{10}O_2$	49.90
P6	1'-Hydroxychavicol acetate	$C_{11}H_{12}O_3$	13.23
P7	Eugenol acetate	$C_{12}H_{14}O_3$	3.45
P8	4-Allyl-1,2-diacetoxybenzene	$C_{13}H_{14}O_4$	11.77
P9	Phytol	$C_{20}H_{40}O$	0.33
P10	Phytol acetate	$C_{22}H_{42}O_2$	0.94
P11	Vitamin E	$C_{29}H_{50}O_2$	0.51
P12	Campesterol	$C_{28}H_{48}O$	0.51
P13	Stigmasterol	$C_{29}H_{48}O$	0.55
P14	β-Sitosterol	$C_{29}H_{50}O$	2.49

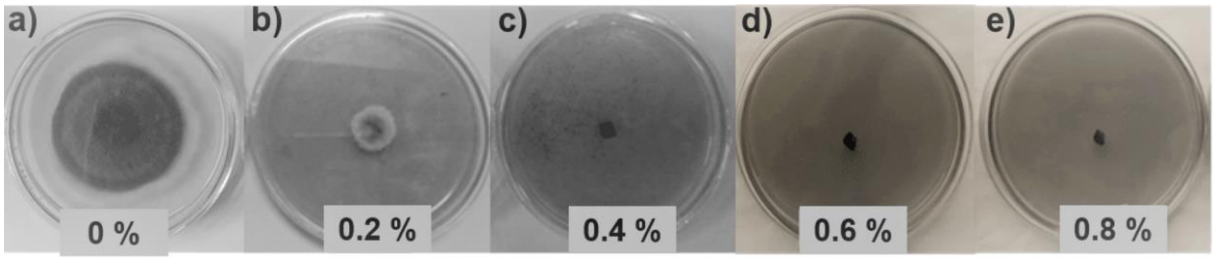

Figure 2: Mycelial growth of *M. oryzae* after 5 days of incubation on PDA added by different concentrations of *Piper betle* extract (a) 0 %, (b) 0.2 %, (c) 0.4 %, (d) 0.6%, and (e) 0.8 %

Table 2: M. oryzae inhibition by *Piper betle* extract at different concentrations

Piper betle extract concentration (%)	Mycelial disc diameters (mm)			*M. oryzae* inhibition (%)		
	3 days	5 days	7 days	3 days	5 days	7 days
0	38.50^a	67.00^a	78.50^a	0.00	0.00	0.00
0.2	15.10^b	19.00^b	23.20^b	60.76	71.67	70.47
0.4	0.00^c	0.00^c	0.00^c	100	100	100
0.6	0.00^c	0.00^c	0.00^c	100	100	100
0.8	0.00^c	0.00^c	0.00^c	100	100	100

In the same column, different letters (e.g., a, b, c) show differences between treatments at $P_{0.05}$

Computation

Enzyme inhibitability

Piper betle-extracted ligands (**P1-P14**) and *M. oryzae* trehalose-6-phosphate synthase (PDB-6JBR) were subjected for the docking-based investigations. The output data is summarised in Table 3 and its visual presentation is projected in Figure 3. It is noticeable that 4-Chromanol, 1'-Hydroxychavicol acetate, Eugenol acetate, and 4-Allyl-1,2-diacetoxybenzene together composing nearly 80 % of the total extract also register DS values of significance regarding their inhibitory systems with 6JBR structure from the *in silico* experiments. The corresponding figures are -12.9, -14.1, -13.1, and -12.5 kcal.mol^{-1} for **P5-6JBR**, **P6-6JBR**, **P7-6JBR**, and **P8-6JBR**, respectively, rather significant to those of other duosystems reported in the literature.[22–24] This means their static stability is predicted to extended certainty. Besides, the RMSD values all under 2 Å predict biologically rigid conformations[25] meanwhile, strong hydrophilic interactions (under -4 kcal.mol^{-1} for total Gibbs free energy, and under 4 Å for average bonding distance) are likely conducive to inducing conformational changes in the protein structures, thus enzymatic malfunction ensuing. Otherwise, although registering a good DS value (-11.8 kcal.mol^{-1}) for **P1-6JBR**, Chavicol only makes up 0.48 % of the total extract composition. In contrast, β-Sitosterol portion is 7.88 % but its corresponding

inhibitory complex, viz. **P2-6JBR**, is formed with a moderate stability given the DS value of -10.2 kcal.mol^{-1}. Therefore, these components are unlikely to contribute to the overall inhibition of *Piper betle* towards *M. oryzae*.

Physicochemical properties

Table 4 summarises QSARIS-based physicochemical properties of the investigated ligands, including molecular mass (amu), polarizability (Å^3) and volume or size (Å) as well as the logP and logS dispersion coefficients. These parameters can be thought to represent pre-docking conditions, i.e. the interactions between the ligands and potential plasmatic components in the polarised media of biological bodies. According to Lipinski's rule of five, **P9-P14** seem not to be suitable for bio-environment applications as their values of logP are over +5 coupling with their prohibitively bulk sizes of over 650 Å; also, **P3** and **P4** are unflavoured given their threshold-closed (4.10 and 4.65) values of dispersion. Although **P1-P2** possess biocompatible mass (under 200 amu) and dispersibility (logP < 3), their insignificant polarisability (under 20) would deter the formation of molecular dielectric moments,[26] thus also unconducive to applications in polarised environments. In contrast, **P5-P8** all register their pronounced bio-compatibility given all factors, including mass (under 150 amu), polarisability (ca. 20 Å^3), dispersibility (logP < 2.5).

Table 3: Molecular docking simulation results for inhibitory complexes between the extracted compounds (**P1-P14**) and the protein 6JBR

Ligand-protein complex			Hydrogen bond			van der Waals
C	DS	RMSD	N	E	D	N
P1-6JBR	-11.8	1.41	3	-6.7	3.82	11
P2-6JBR	-10.2	1.06	2	-2.1	3.62	9
P3-6JBR	-7.4	1.16	0	-	-	26
P4-6JBR	-8.4	0.71	0	-	-	30
P5-6JBR	-12.9	1.15	3	-6.2	3.11	7
P6-6JBR	-14.1	1.95	4	-4.3	3.39	14
P7-6JBR	-13.1	1.25	3	-6.3	3.03	13
P8-6JBR	-12.5	1.56	3	-4.2	3.42	14
P9-6JBR	-10.9	1.88	2	-1.6	3.11	15
P10-6JBR	-9.7	1.13	2	-7.3	3.05	20
P11-6JBR	-9.8	1.21	2	-1.7	4.39	22
P12-6JBR	-9.2	1.23	1	-0.8	3.26	17
P13-6JBR	-10.0	1.01	2	-0.8	2.69	21
P14-6JBR	-10.4	1.23	2	-2.4	2.84	18

C: Complex; **DS**: Docking score energy (kcal.mol^{-1}); **RMSD**: Root-mean-square deviation (Å)

N: Number of interactions; **E**: Total Gibbs free energy (kcal.mol^{-1}); **D**: Average bonding distance (Å)

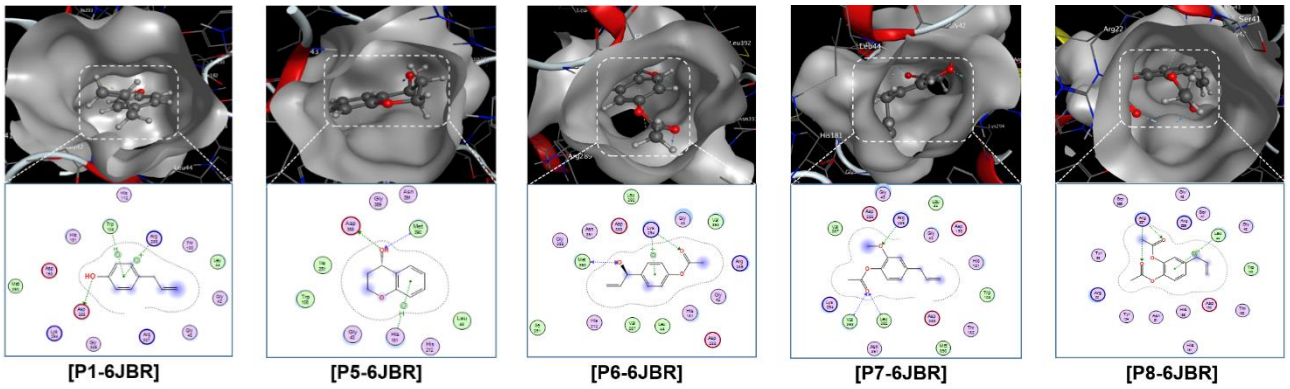

Figure 3: Visual presentation and in-pose interaction map of ligand-6JBR inhibitory complexes: **P1-6JBR**, **P5-6JBR**, **P6-6JBR**, **P7-6JBR**, **P8-6JBR**

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Table 4: Physicochemical properties of studied compounds (**P1-P14**)

Compound	Mass (amu)	Polarisability ($Å^3$)	Size (Å)	Dispersion coefficients	
				LogP	LogS
P1	137.3	17.8	220.1	2.54	-2.10
P2	164.4	18.6	261.5	2.23	-2.05
P3	204.5	25.3	380.3	4.10	-4.21
P4	204.4	28.0	416.1	4.65	-4.39
P5	150.5	19.3	206.9	1.97	-1.18
P6	192.6	22.1	282.7	1.69	-1.23
P7	206.5	24.8	325.0	2.23	-2.31
P8	234.7	25.7	350.8	2.17	-2.42
P9	296.6	39.4	580.3	6.26	-5.10
P10	338.9	43.2	650.6	7.03	-5.06
P11	430.8	54.4	769.4	8.49	-7.30
P12	400.5	48.4	670.9	8.02	-7.01
P13	412.5	51.9	695.2	8.97	-7.19
P14	414.8	52.4	705.7	8.90	-7.24

Conclusions

This study suggests a well-established inhibition mechanism regarding *Piper betle* extract towards *Magnaporthe oryzae*. GC-MS characterisation determines 4-Chromanol (**P5**), 1'-Hydroxychavicol acetate (**P6**), Eugenol acetate (**P7**), and 4-Allyl-1,2-diacetoxybenzene (**P8**) making up the majority of *Piper betle* extract composition, ca. 80 %. Bio-assays provide an experimental evidence of a total antifungal effect towards *M. oryzae*, i.e. complete inhibition (100 %). Docking-based simulation confirm the significant static stability of **P5-6JBR** (DS -12.9 $kcal.mol^{-1}$; RMSD 1.25 Å), **P6-6JBR** (DS -14.1 $kcal.mol^{-1}$; RMSD 1.95 Å), **P7-6JBR** (DS -13.1 $kcal.mol^{-1}$; RMSD 1.25 Å), and **P8-6JBR** (DS -12.5 $kcal.mol^{-1}$; RMSD 1.56 Å). QSARIS analysis exceptionalises the physicochemical compatibility of the potential ligands, aka. **P5** (mass 150.5 amu; polarisability 19.3 $Å^3$, logP 1.97), **P6** (mass 192.6 amu; polarisability 22.1 $Å^3$, logP 1.69), **P7** (mass 206.5 amu; polarisability 24.8 $Å^3$, logP 2.23), and **P8** (mass 234.7 amu; polarisability 25.7 $Å^3$, logP 2.17). The results would encourage further in-width computational researches, e.g. on other protein structures, or in-depth experimental investigations, e.g. on isolated inhibition to validate the mechanism proposed.

Acknowledgments

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