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Anti-infective potential of the endophytic fungi associated with *Allium cepa* supported by metabolomics analysis and docking studies

Abstract

Endophytic fungi are known to be a rich source for anti-infective drugs. In our study, *Allium cepa* endophytic fungi were investigated for the first time using the OSMAC approach. Three isolates, *Penicilliun crustosom*, *Asperigullus* sp. and *Penicillium griseofulvum* were identified. Their extracts revealed potential activity against MRSA, *Klebsiella pneumonia*., and *candida albicans*. Additionally, the crude ethyl acetate extract of *Penicillium griseofulvum* was investigated using LC-HR-ESI-MS. The metabolic profiling revealed the presence of polyketides, macrolides, phenolics, and terpenoids. Furthermore, *in silico* molecular docking study was carried out to predict which compounds most likely responsible for the anti-infective activity.

Keywords

Antimicrobial potential; *Allium*; Metabolic profiling; *Peniciilum*; *Aspergillus*; OSMAC; molecular docking

Experimental:

Plant material collection

Healthy fresh roots, stems and leaves (white and green) of *Allium cepa* were collected from the botanical garden of Department of Botany and Microbiology, Faculty of Science, Minia University, Minia, Egypt. The studied plant was identified by Prof. Naser Barakat, Botany and Microbiology Department, Faculty of Science, Minia University, Minia, Egypt.

Endophytic fungal isolation

Endophytic fungal isolation of *Penicillium griseofulvum, Aspergillus sp*, and *Penicillium crustosum* have been carried out in our investigation regarding isolation of endophytic fungi from *Allium cepa.*

Molecular identification and phylogenetic analysis

Taxonomic identification of the isolated fungal strain recovered from *Allium cepa* leaves (D2, C1), also from diseased *Allium cepa* (P2) was achieved by DNA amplification and sequencing of the fungal internal transcribed spacer (ITS) region using the universal primers ITS1 and ITS4 (El-Hawary *et al.* 2016). The phylogenetic distance was inferred by the maximum likelihood method based on the Kimura two-parameter model (Kimura 1980). The tree with the highest log likelihood (14991536) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree (s), for the heuristic search, were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 344 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016).

Fungal fermentation and extract preparation

The isolated subcultured *Penicillium griseofulvum (LCEF10), Aspergillus sp. (LCEF6), Penicillium crustosom(LCPF2)* were fermented in 4 different media namely, potato dextrose broth. (PDA) consisting of 200 g potato, 20 g glucose and 1000 ml water, modified potato dextrose broth (PDB/Y) which is PDA added with yeast extract to pH 5.5, capeks (CZ) consisting of 20 g agar ,30g cane sugar,1g dipotassium phosphate, 0.5g magnesium sulfate, 0.5g potassium chloride, 0.01g iron sulfate with 1000ml water, Rice media containing 20g rice with 1000 ml water. The flasks were incubated under static conditions at 28°c for 7 days. At the end of fermentation, ethyl acetate (300 ml) was added to each flask for stopping fermentation. The fungal mycelia together with the culture broth were subjected to ultrasound-assisted extraction with ethyl acetate (300 ml) three times to afford 4 culture broth ethyl acetate extracts (E1, E2, E3, E4) and four fungal mycelia ethyl acetate extracts (M1, M2, M3, M4) for PDB, PDB/Y, RICE and CZ. The extracts were then concentrated using rotary evaporator (Buchi Rotavapor R300: BUCHI Labortechnik, Essen, Germany)

Metabolomics analysis

Metabolomic profiling of the crude extracts of *Penicillium griseofulvum* was carried out according to Abdelmohsen et al. (Abdelmohsen *et al.* 2014) using an Acquity Ultra Performance Liquid Chromatography system coupled to a Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer (Waters, Milford, USA). Additionally, Ms converter software was utilized in order to convert the raw data into divided positive and negative ionization files. The obtained files were then subjected to the data mining software MZmine 2.10 (Okinawa Institute of Science and Technology Graduate University, Japan) for deconvolution, peak picking, alignment, deisotoping, and formula prediction. The MarinLit (http://pubs.rsc.org/marinlit/), DNP (http://dnp.chemnetbase.com/faces/chemical/ChemicalSearch. xhtml), and METLIN (http://metlin.scripps.edu/index.php) databases were successfully used for dereplication of the detected metabolites.

Test micro-organisms

The micro-organisms used during the antimicrobial study included three human pathogenic microorganisms (MRSA, *Klebsiella pneumonia*, and *Candida albicans*). All micro-organisms were obtained from Department of Microbiology, Faculty of Pharmacy, Deraya University, Minia, Egypt.

Antimicrobial assay

Antimicrobial screening of the isolated endophytic fungi was carried out according to Zhang et al. (Zhang *et al.* 2009), which qualitatively allows for a rapid selection of the most potent endophytes. Each isolated strain was cultivated on PDA in Petri dishes for 7 days at 30 °C. Additionally, using sterile cork borer, disks (5 mm in diameter) were cut from the PDA plate, and transferred to the surface of Petri dishes which was previously spread with the tested micro-organisms using different media, either PDA for inoculation of fungi or Nutrient Agar (Himedia Laboratories, India) for bacteria. The Petri dishes were then incubated at 37 °C for 24 h to allow bacterial growth and at 30 °C for 72 h for fungal growth. The antimicrobial potential was performed by measuring the diameter of inhibition zones in millimeters. Three independent experiments were carried out. IC_{50} values were determined as the concentration that produces 50% inhibition of the growth of cells and were calculated by GraphPad Prism 5 (Version 5.01, GraphPad Software, San Diego, CA, USA).

Molecular Docking Method

Cytidine deaminase (CDA) X-ray crystal structure was downloaded from the Protein Data Bank (PDB ID: 6K63) (Liu 2019), corrected and 3D protonated using amber10:EHT forcefield of Molecular Operating Environment (MOE 2014.0901) software. The best quality chain A was selected to perform molecular docking followed by water molecules removal and zinc ion retention as a crucial co-factor. The binding site was selected through dummies as alpha centers then molecular docking was performed using Triangle Matcher, London dG, GBVI/WSA as the placement, rescoring function 1 and 2, respectively as the docking algorithm.

Statistical Analysis

The results were expressed as the means ± SEM of the indicated number of experiments ($n \geq 3$). The statistical significance of differences between means was established by ANOVA with Duncan's post hoc tests. *P* values <0.05 were considered to indicate statistical significance.

Results and discussion

In this work, three endophytic fungi strains, *P. crustosom*, *Asperigullus* sp., and *P. griseofulvum*, were recovered from the *A. cepa* leaves. The three fungal strains were identified morphologically in addition to phylogenetic analysis and fermented on four

different media (PDA, PDB/Y, Capeks, and Rice media), followed by extraction with ethyl acetate. Moreover, the anti-infective potential (**Table S1**) of the total ethyl acetate extracts of the three *A. cepa* endophytic fungi strains *P. grisefoulvm*, *P. crustosom*, and *Aspergillus* sp. were evaluated against two human pathogenic bacteria MRSA, *Klebsiella pneumonia*, and one human pathogenic fungus *Candida albicans* using agar disc diffusion assay. The total extract of *P. grisefoulvm* showed the highest activity against *Klebsiella pneumonia* with IC_{50} value of 3.8 µg/mL, followed by *P. crustosom*, and *Aspergillus* sp with IC_{50} values of 7.4 and 73.7 µg/mL, respectively. Additionally, the total extract of *Aspergillus* sp. was the most potent one against MRSA with IC_{50} Value of 1.1 µg/mL, followed by the total extracts of *P. crustosom* and *P. grisefoulvm* with IC_{50} values of 8.8, 12.1 µg/mL, respectively. On the other hand, the total extract of *Aspergillus* sp. was the most active one against *candida albicans* with IC_{50} value of 13.8 µg/mL followed by the total extracts of *P. grisefoulvm*, *P. crustosom* with IC_{50} values of 17.6 and 18.2 µg/mL, respectively.

On the other hand, Metabolomics analysis of the crude extract of *P. griseofulvum* using LC–HR–ESI–MS for dereplication purposes **(Fig. S1 and S2)** has resulted in the identification of a range of varied secondary metabolites that were dominated by terpenoides and polyketides. The detected compounds (**Fig. 1, Table S2**) were identified by coupling MZmine with some databases, namely Marinlit and DNP. In view of that, the mass ion peak at *m/z* 253.1071 for the suggested molecular formula $C_{13}H_{18}O_5$ was identified as Striatisporin A (**1**) which was previously isolated from *P. striatisporum* (Stewart *et al.* 2005). Another mass ion peak at *m/z* 267.1228 for the molecular formula $C_{14}H_{20}O_5$ was identified as Phomaligadione A **(2).** This polyketide was previously obtained from *P. wasabiae* (Zhang *et al.* 2020). Whereas that at *m/z* 289.142 for the molecular formula $C_{17}H_{20}O_4$ was identified as BE-25327 **(3)** that was previously isolated as anti-osteoprotic agent from *P. purpurogenum f25327* (Maurya *et al.* 2008) (Maurya, Singh et al. 2008). Additionally, the mass ion peak at *m/z* 289.1071, corresponding to the proposed molecular formula $C_{16}H_{18}O_5$ was identified as Dehydrocurvularin **(4)**. It was formerly isolated from *Penicillium* sp. *a-5-11* (Xie *et al.* 2009, Iqbal *et al.* 2017). ML-236C **(5)** was dereplicated at *m/z* 289.179 for the suggested molecular formula $C_{18}H_{26}O_3$, previously obtained from *P. citrinum* (Endo *et al.* 1976), it exhibited potent anti-hypercholestermeic potential. Moreover, the mass ion peak at *m/z* 291.194 and the predicted molecular formula $C_{18}H_{26}O_3$ was identified as potent anti-inflammatory agent namelyTanzawaic acid C **(6),** which was previously isolated from *P. citrinum* (Kuramoto *et al.* 1997).

As well as, the mass ion peak at *m/z* 291.122 and the molecular formula $C_{16}H_{20}O_5$ was identied as curvularin (**7**), which was previously obtained from *Penicillium* sp. (Kobayashi *et al.* 1988). On the other hand, the mass ion peaks at *m/z* 305.138, 321.133, and 323.1489 in accordance with the molecular formulas $C_{17}H_{22}O_5$, $C_{17}H_{22}O_6$, and $C_{17}H_{24}O_6$ were recognized as Eremofortin A, C, and D, respectively (**8, 9, 10**). Those eremophilane-sesquiterpenes derivatives were previously isolated from *P. roqueforti.* (Moreau *et al.* 1980). Furthermore, the mass ion peak at *m/z* 323.1852 and the suggested molecular formula $C_{18}H_{28}O_5$ was identified as Hynapene-A (**11**), which was earlier isolated from *Penicillium* sp. FO-1611. (Tabata *et al.* 1993). Another compound was identified as A-26771B (**12**) on account of the observed mass ion peak *m/z* 381.1903 and in accordance with the molecular formula $C_{20}H_{30}O_7$. This macrocyclic lactone was previously isolated from *P. turbatum* (Michel *et al.* 1977).

Moreover, the mass ion peak at *m/z* 385.2359 and the suggested molecular formula $C_{24}H_{32}O_4$, was identified as 6-Farnesyl-5,7-dihydroxy-4-methylphthalide (**13**), which was previously isolated from *P. brevicompactum* (Colombo *et al.* 1978). 4a,5-Dihydrocompactin (**14**) was dereplicated at *m/z* 393.2624, which in agreement with the molecular formula $C_{23}H_{36}O_5$. This terpenoid was previously obtained from *P. citrium* (Lam *et al.* 1981). The mass ion peak at *m/z* 408.2747, and the molecular formula $C_{23}H_{37}NO_5$ was identified as Ro-09-1545 (**15**) which was formerly obtained from *P. sclerotiorum* (MATSUKUMA *et al.* 1992)

Furthermore, Austinoneol A (**16**) was dereplicated at *m/z* 415.2099 and in accordance with the molecular formula $C_{24}H_{36}O_6$, which was previously isolated from *Penicillium* sp. (Santos and Rodrigues-Filho 2003). Likewise, the mass ion peak at *m/z* 417.226 and the molecular formula $C_{24}H_{32}O_6$ was identified as Verrucosidin (**17**), which was previously isolated from *P. verrucosum* (Burka *et al.* 1983). Additionally, the mass ion peak at *m/z* 443.2428, in keeping with the molecular formula $C_{26}H_{36}O_6$, was dereplicated as the meroterpene preaustinoid A **(18)**, which was formerly purified from *Penicillium* sp. (Fill *et al.* 2007). Citreohybridone B (**19**) was characterized at *m/z* 515.2632 and the molecular formula $C_{29}H_{38}O_8$. This compound was previously isolated from *P. citreoviride* b hybrid strain ko 0031 (Kosemura *et al.* 1991). The mass ion peak at *m/z* 581.2851 with the molecular formula $C_{30}H_{39}N_5O_7$ was identified as JBIR-114 (**20**) previously isolated from *Penicillium* sp. (Fill *et al.* 2016). Finally the mass ion peak at *m/z* 239.0902, and the

molecular formula $C_{12}H_{14}O_5$ was identified as 4-(4-Formyl-2-methoxy phenoxy) butanoic acid (**21**), which was previously isolated from *Penicillium* sp. GT6105 (Li *et al.* 2007).

In order to explain the outstanding *in vitro* biology results, *in silico* molecular docking was performed using cytidine deaminase enzyme (CDA) isolated from *Klebsiella pneumoniae.* as a potential target. CDA is a zinc-dependent enzyme that catalyzes the oxidative deamination of cytidine and 2`-deoxycytidine to form uridine and 2`-deoxyuridine, respectively (S. Xiang 1995, A. Marx 2015, A. Moro-Bulnes 2019, Wei Liu 2019). CDA protein crystal structure was downloaded from the Protein Data Bank (PDB ID: 6K63) (Liu 2019) and compounds **1-21** were evaluated for their binding potential to its binding site through binding energy score and poses to maintain the essential interactions as shown in table **S3**.

As shown in table **S3**, the binding energy score ranges from -13.86 to -6.00 Kcal/mol out of which striatisporin A (**1),** hynapene-A **(11)**, (-)-A-26771B (**12)** and 4-(4-formyl-2-methoxy phenoxy) butanoic acid (**21)** showed the lowest energy values. Knowing that zinc ion is co-ordinated with Cys129, Cys132 and His102 at CDA catalytic domain hence, binding to those residues would hinder its catalytic activity (N. Mejlhede 2000, Wei Liu 2019). All compounds **1-21** were capable of interacting with one or more of the three crucial amino acid residues of the catalytic domain. The promising compounds **1**, **11**, **12** and **21** binding conformations inside the binding site of CDA were illustrated in Fig 1 and 2. As demonstrated in **Fig. S3** and **S4**, the terminal deprotonated carboxylic acid moiety of **1**, **11**, **12** and **21** co-ordinated with zinc ion through either one bond at **11** and **21** or two bonds at **1** and **12**. Additionally, the carboxylate moiety formed H-bond with the essential Cys129 as H-acceptor and Cys132 as H- donor with a distance range 2.80-3.43 Å. Furthermore, compounds **1** and **11** formed H- bond with the conserved Glu104 and Glu91, respectively through their hydroxyl moieties in addition to another H-bond formed between the hydroxyl group of **11** and Asn89. Similarly, the olefinic group of **12** and aldehydic carbonyl of **21** founded hydrophobic interaction with Phe 71 and H- bond with Met97, respectively.

It was noticed that the majority of the compounds were able to co-ordinate with zinc ion except compounds BE-25327 **(3)**, *αβ*-dehydrocurvularin **(4)** and Ro-09-1545 **(15)** as shown in **Fig. S5**. Their inability to coordinate with zinc ion might explain their possible low antibacterial activity through CDA inhibition compared to the other compounds. Moreover, their calculated moderate binding energy score might support this assumption giving -6.72, -7.65 and -9.32 Kcal/mol for **3, 4** and **15**, respectively. However, they

managed to form a hydrophobic interaction with the essential His102 moiety which suggested a potential CDA inhibition.

Table S1. *In vitro* antimicrobial potential of the ethyl acetate extracts of *P. crustosom*, *Asperigullus* sp. and *P. griseofulvum* against MRSA, *Klebsiella pneumoniae*, and *candida albicans*

Extracts	MRSA IC_{50} (µg/mL)	*Klebsiella pneumoniae* IC_{50} (µg/mL)	*Candida albicans* IC_{50} (µg/mL)
Ethyl acetate extract of *P. crustosom*	8.87	7.44	18.28
Ethyl acetate extract of *Asperigullus* sp.	1.11	73.78	13.84
Ethyl acetate extract of *P. griseofulvum*	12.10	3.89	17.66

Table S2. A list of the dereplicated metabolites from the investigated extract of *P. grisefoulvm*.

No	***m/z***	**Molecular formula**	**Rt.**	**Molecular weight**	**Name**	**Source**	**References**
1	253.1071	$C_{13}H_{18}O_5$	3.80	254.1144	Striatisporin A	*P. striatisporum*	(Stewart, Capon et al. 2005)
2	267.1228	$C_{14}H_{20}O_5$	3.15	268.1301	Phomaligadione A	*P. wasabiae*	(Zhang, Fasoyin et al. 2020)
3	289.1425	$C_{17}H_{20}O_4$	5.54	288.1352	BE-25327	*P. purpurogenum f25327*	(Maurya, Singh et al. 2008)
4	289.1071	$C_{16}H_{18}O_5$	5.18	290.1144	Dehydrocurvularin	*Penicillium* sp. *a-5-11*	(Iqbal, Khan et al. 2017)
5	289.1799	$C_{18}H_{26}O_3$	5.80	290.1872	ML-236C	*P. citrinum,*	(Endo, Kuroda et al. 1976)

6	291.1949	$C_{18}H_{26}O_3$	8.86	290.1876	Tanzawaic acid C		(Kuramoto, Yamada et al. 1997)
7	291.1229	$C_{16}H_{20}O_5$	5.62	292.1302	(-)-Curvularin	*Penicillium* sp. *a-5-11*	(Kobayashi, Hino et al. 1988)
8	305.1382	$C_{17}H_{22}O_5$	5.46	306.1455	Eremofortin A	*P. roqueforti*	(Moreau, Lablache-Combier et al. 1980)
9	321.1331	$C_{17}H_{22}O_6$	4.94	322.1404	Eremofortin C		(Moreau, Lablache-Combier et al. 1980)
10	323.1489	$C_{17}H_{24}O_6$	4.73	324.1561	Eremofortin D		
11	323.1852	$C_{18}H_{28}O_5$	5.56	324.1925	Hynapene-A	*Penicillium sp. FO-1611*	(Tabata, Tomoda et al. 1993)
12	381.1903	$C_{20}H_{30}O_7$	3.88	382.1976	(-)-A-26771B	*P. turbatum*	(Michel, Demarco et al. 1977)
13	385.2359	$C_{24}H_{32}O_4$	12.66	384.2286	6-Farnesyl-5,7-dihydroxy-4-methylphthalide	*P. brevi-compactum*	(Colombo, Gennari et al. 1978)
14	393.2624	$C_{23}H_{36}O_5$	9.53	392.2551	4a,5-Dihydroco-mpactin	*P. citrium*	(Lam, Gullo et al. 1981)
15	408.2747	$C_{23}H_{37}NO_5$	4.33	407.2674	Ro-09-1545	*P. sclerotiorum*	(MATSUKUMA, OHTSUKA et al. 1992)
16	415.2099	$C_{24}H_{36}O_6$	6.75	414.2027	Austinoneol A	*Penicillium* sp.	(Santos and Rodrigues-Filho 2003)
17	417.226	$C_{24}H_{32}O_6$	6.18	416.2183	Verrucosidin	*P. verrucosum*	(Burka, Ganguli et al. 1983)
18	443.2428	$C_{26}H_{36}O_6$	7.86	444.2501	Preaustinoid A	*Penicillium* sp.	(Fill, Pereira et al. 2007)
19	515.2632	$C_{29}H_{38}O_8$	7.77	514.2559	Citreohybridone B	*P. citreoviride b hybrid strain ko 0031*	(Kosemura, Matsunaga et al. 1991)
20	581.2851	$C_{30}H_{39}N_5O_7$	8.55	582.2924	JBIR-114	*Penicillium* sp.	(Fill, Pallini et al. 2016)

21	239.0902	$C_{12}H_{14}O_5$	3.50	238.083	4-(4-Formyl-2-methoxy phenoxy) butanoic acid	*Penicillium* sp. GT6105	(Li, Yao et al. 2007)

Table S3. Molecular docking results of compounds **1-21** using *Klebsiella pneumoniae* CDA enzyme (PDB ID: 6K63).

Compounds	Binding energy score (Kcal/mol)	Crucial residues interaction	No. of zinc binding group
1	-12.17	Glu104 (H-donor), Cys129 (H- acceptor), Cys132 (H-donor)	1
2	-8.25	His102	1
3	-6.72	His102 (arene-arene), Glu104 (H-donor)	None
4	-7.65	His102 (arene-H), Ala103 (H-donor)	None
5	-9.08	Cys129 (H- acceptor), Cys132 (H-donor)	1
6	-11.21	Cys129 (H- acceptor), Cys132 (H-donor)	1
7	-7.73	His102 (arene-arene)	1
8	-8.64	Cys129 (H- acceptor)	1
9	-8.91	Cys129 (H- acceptor)	1
10	-9.23	Cys129 (H- acceptor), Cys132 (H-donor)	1
11	-12.32	Asn89 (H-acceptor), Glu91 (H-donor), Cys129 (H-acceptor), Cys132 (H-donor)	1
12	-13.86	Phe71 (arene-H), Cys129 (H- acceptor), Cys132 (H-donor)	1
13	-10.64	His102 (arene-H), Glu104 (H-donor), Cys129 (H-	1

		acceptor), Cys132 (H-donor)	
14	-9.39	Cys129 (H- acceptor), Cys132 (H-donor)	1
15	-9.32	Met 97 (2H-donor), Gln98 (H-acceptor), His102 (arene-H, H-acceptor)	None
16	-10.27	Asn89 (H-acceptor), Cys129 (H- acceptor)	1
17	-8.84	Cys129 (H- acceptor), Cys132 (H-donor)	1
18	-7.23	Met97 (H-donor)	1
19	-7.89	Cys132 (H-donor)	1
20	-8.81	His102 (arene-H), Cys129 (H-donor)	1
21	-12.66	Met97 (H-donor), Cys129 (H- acceptor), Cys132 (H-donor)	1

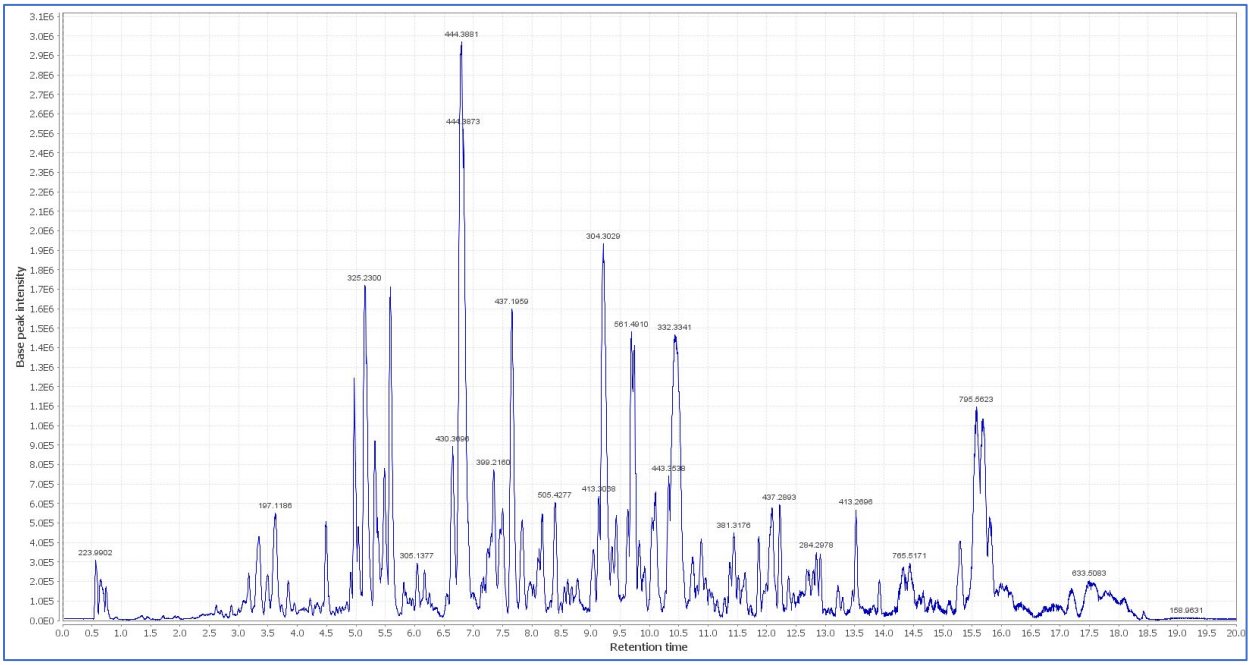


Fig. S1: Total ion chromatogram for the ethyl acetate extract of *P. grisefoulvm* on positive ionization mode.

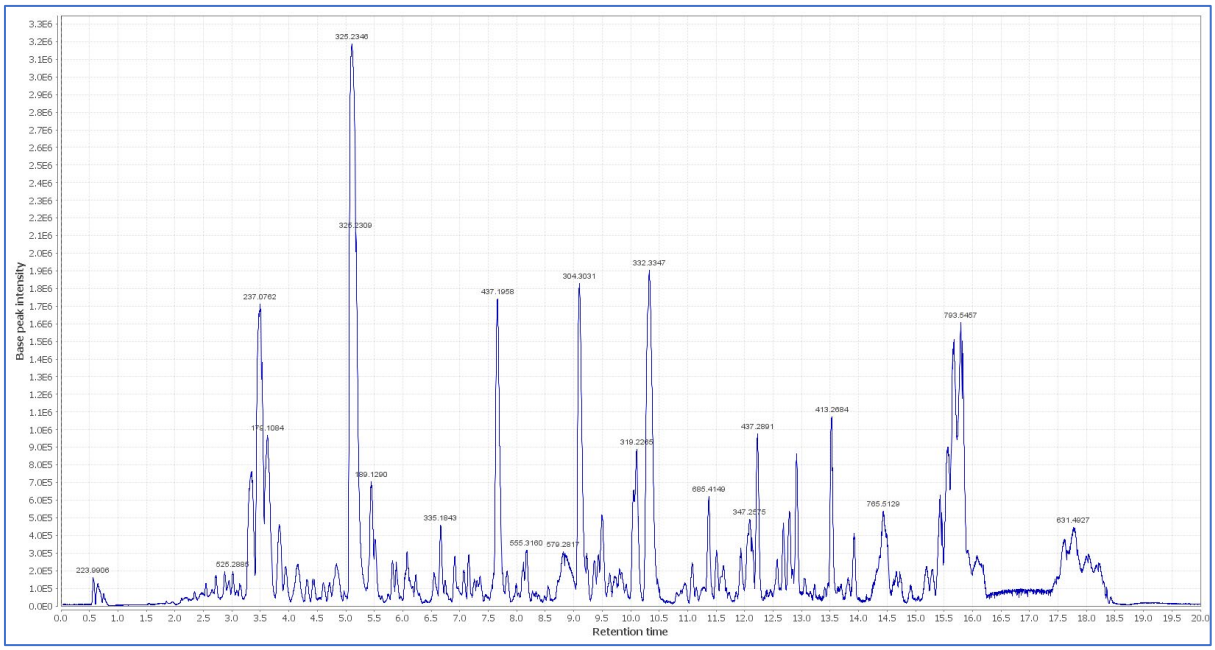


Fig. S2: Total ion chromatogram for the ethyl acetate extract of *P. grisefoulvm* on negative ionization mode.

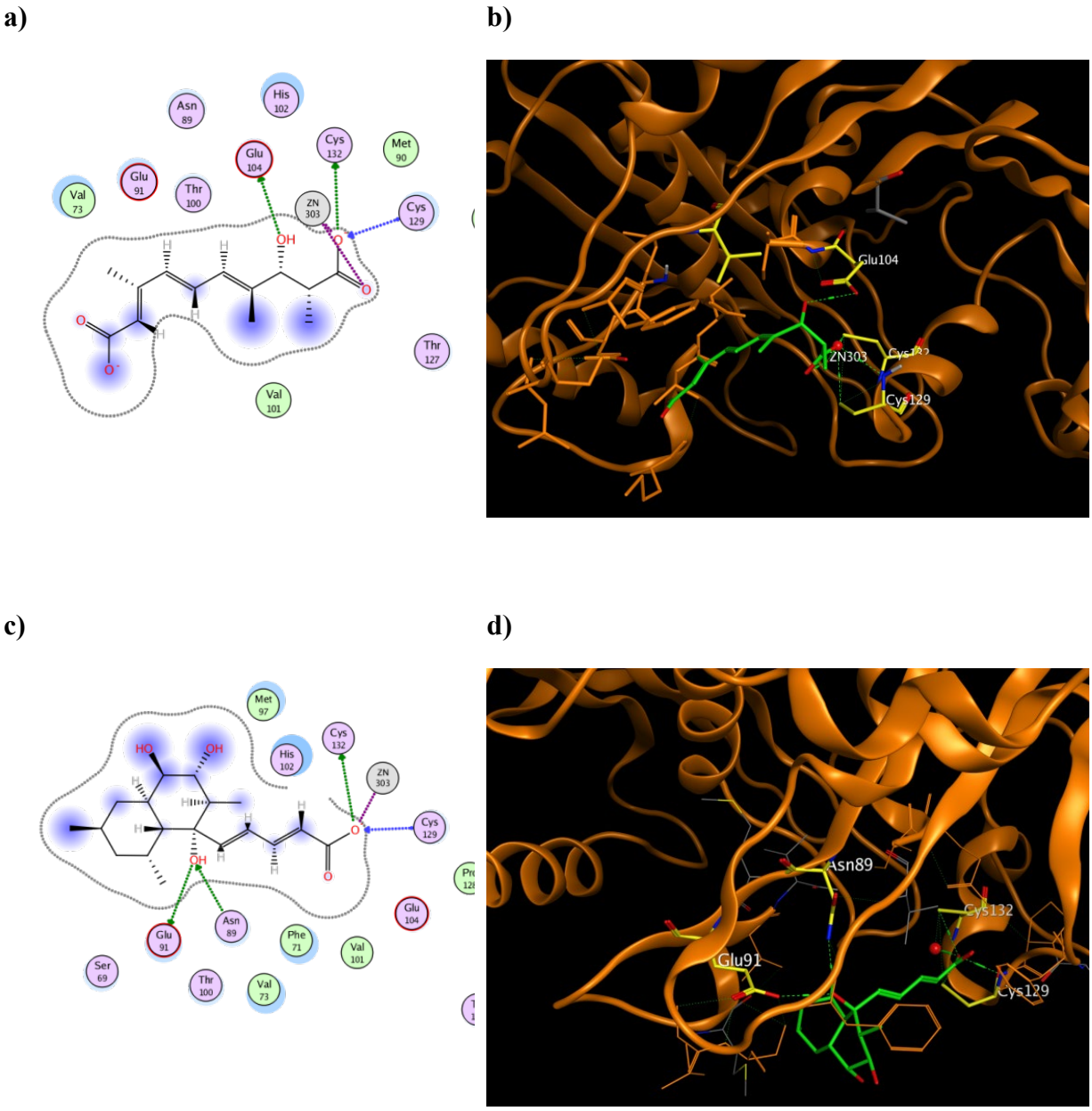
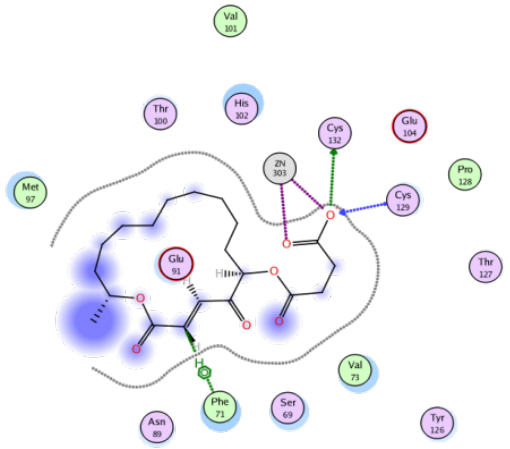
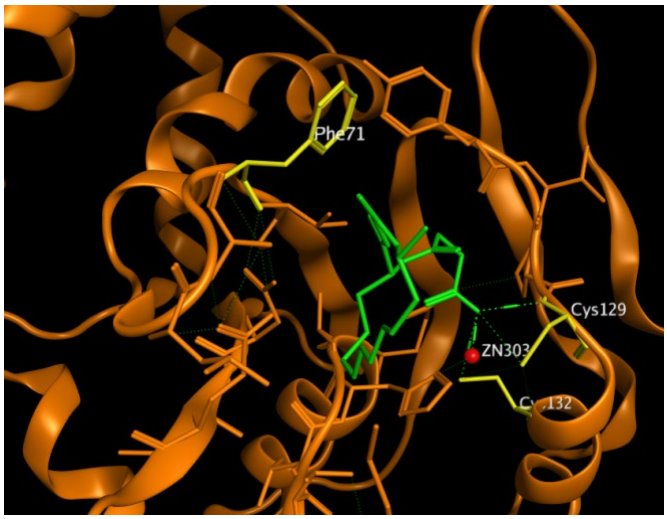


Fig. S3. The 2D and 3D graphical presentation of compounds **1** (a,b) and **11** (c,d) molecular docking using PDB 6K63 where both compounds were shown as green stick model with the interacting residues were marked in yellow. The hydrogen bonds were presented as green dotted line.

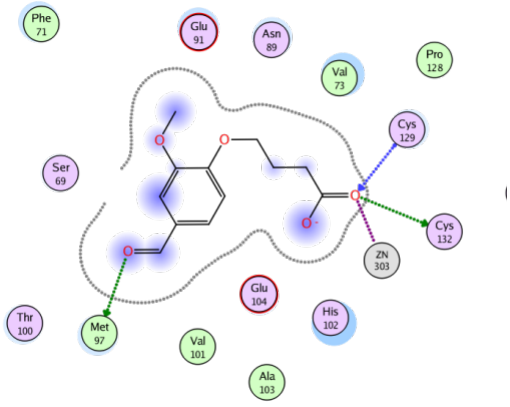
a)



b)



c)



d)

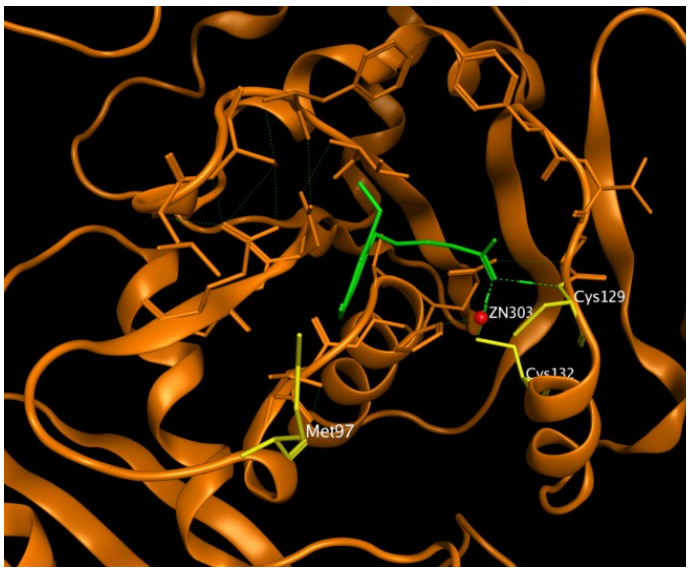


Fig. S4. The 2D and 3D graphical presentation of compounds **12** (a,b) and **21** (c,d) molecular docking using PDB 6K63 where both compounds were shown as green stick model with the interacting residues were marked in yellow. The hydrogen bonds were presented as green dotted line.

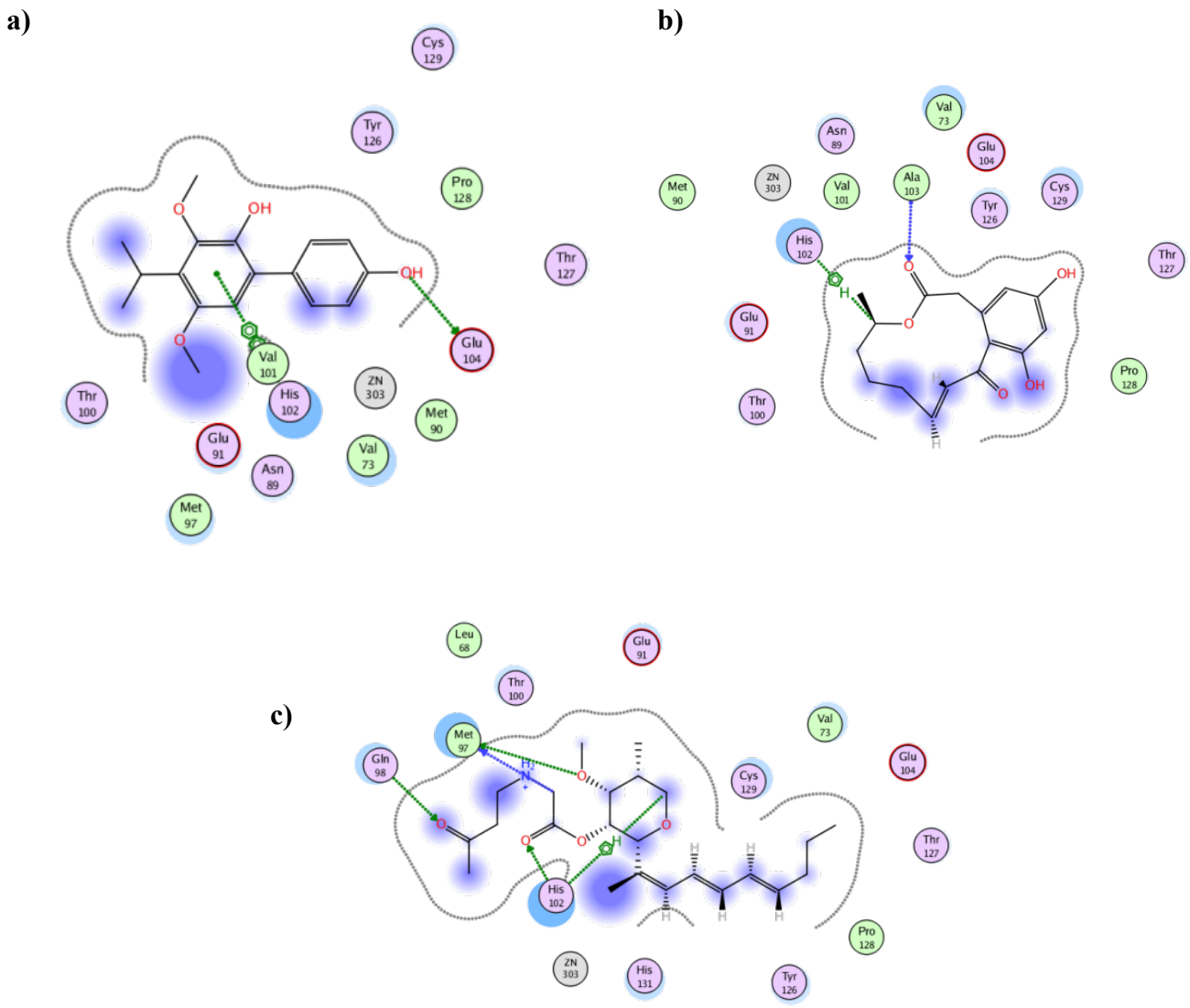


Fig. S5. The 2D molecular docking interaction of compounds **3** (a), **4** (b) and **15** (c) using PDB ID 6K63

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