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Earth and
Environmental Science

PAPER • **OPEN ACCESS**

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To cite this article: H Husnawati *et al* 2024 *IOP Conf. Ser.: Earth Environ. Sci.* **1359** 012138

IOP Conf. Series: Earth and Environmental Science **1359** (2024) 012138 doi:10.1088/1755-1315/1359/1/012138

Standardization of *Phomopsis* sp. extract and prediction of anti breast cancer activity from the main compound through molecular docking

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Abstract. Endophytic fungi isolated from Indonesian soursop (*Annona muricata*) leaves, which have the potential to treat breast cancer, have been successfully isolated since 2016. Identification based on the Internal Transcribed Spacer (ITS) gene revealed that fungi close to *Phomopsis sp*. The fungi were stored, subcultured, and extracted with ethyl acetate for *in vitro* and *in vivo* studies. To be registered as a medicinal ingredient and meet the requirements of the Indonesian Food and Drug Supervisory Agency (BPOM, Badan Pengawas Obat dan Makanan), it is necessary to ensure the standardization, safety, and quality of the *Phomopsis* extract. The results showed that *the Phomopsis* extract met the BPOM requirements for organoleptic, water, and solvent content, microbial and heavy metal contamination, total aflatoxin, and metabolite content. Analysis of the *Phomopsis* extract content also showed that the compounds in the two production batches were stable, with the highest relative abundance of 7-hydroxycoumarin. *In silico* studies with molecular docking show that the affinity energy of 7-hydroxycoumarin docking with the estrogen receptor alpha (ERα) and thymidine kinase 1 (TK1) has a lower negativity value than the comparison ligand (-6.7 and -6.8 kcal/mol).

1. Introduction

Annona muricata (soursop) is an alternative medicinal plant that has been widely studied for its potential in the treatment of several diseases, including breast cancer. [1]. Soursop fruit is Indonesia's national agricultural commodity; therefore, excessive use of soursop leaves can be a problem because it can disrupt soursop fruit production. Additionally, extraction using soursop leaves raw materials requires harvesting time and suitable soil conditions. Therefore, we explored the anticancer potential of endophytic organisms present in soursop leaves.

Endophytes are microorganisms that live symbiotically with host plants in colonies [2]. Endophytes can be isolated and grown in suitable growth media for cultivation in the laboratory. Symbiosis between

endophytic organisms and their hosts can cause some genetic material from the host to be transferred to certain endophytic organisms, resulting in the production of the same secondary metabolites [3]. Many endophytic organisms are present in soursop leaves, including bacteria and fungi [4]. Based on previous studies, several endophytic fungi with anticancer activity (*in vitro* and *in vivo*) were found in soursop leaves [5]. The solvent generally used for the extraction of endophytic fungi is ethyl acetate [6].

Analysis using an Internal Transcribed Spacer (ITS) showed that the endophytic fungus with anti-breast cancer activity, isolated from *Annona muricata* leaves from Indonesia, was *Phomopsis* sp. [5]. The ethyl acetate extract of *Phomopsis* can inhibit MCF-7 cells at an IC_{50} value < 20 ppm [5]. A previous *in vivo* study showed the ability of *Phomopsis* extract to significantly reduce the volume of breast tumors in *Sprague-Dawley* rats at an optimal dose of 20 mg/kgBW [7]. However, the molecular mechanisms underlying the anti-breast cancer effects of this extract remain unclear.

Many pathways and proteins play roles in breast cancer. Some of these include human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and thymidine kinase 1 (TK1), which play a role in proliferation and the tumor cell cycle [8, 9]. An *in silico* study analyzing the molecular interactions between compounds in *the Phomopsis* extract and HER2 has been conducted [10].

The *Phomopsis* fungus was stored and cultured in a microbiology laboratory. This fungus has been used several times in *in vitro* and *in vivo* breast cancer research. To proceed to advanced testing (clinical trials), this extract must be registered and meet the Food and Drug Supervisory Agency (BPOM) requirements. Therefore, it is necessary to standardize the production process and metabolite content in the extract, as well as the safety and quality of the *Phomopsis* extract. In addition, it is necessary to carry out molecular analyses related to the prediction of anti-breast cancer mechanisms from the main compounds in the extract. The aim of this study was to standardize the *Phomopsis* ethyl acetate extract according to BPOM regulations and to predict the anti-breast cancer mechanism of the main compound through molecular docking (MD) with ER and TK1.

2. Method

Phomopsis endophytic fungi from Indonesian *Annona muricata* were stored in the Indonesian Culture Collection - National Research and Innovation Agency (InaCC - BRIN), Bogor, Indonesia (Figure 1a). Before use, the fungi were first rejuvenated in yeast malt media.

2.1. Extraction process

Phomopsis was inoculated on yeast malt agar (YMA) and incubated for 5 – 7 days at room temperature (25±3 ºC). *The* culture was then transferred to 2 liter yeast malt broth (YMB), incubated for 21 days, and shaken at 100 rpm at room temperature (Figure 1b). After 21 days, the *Phomopsis* was harvested and extracted using ethyl acetate through a maceration process. The top fraction of the mixture was then poured into a flask. This fraction was then concentrated using a rotary vacuum evaporator at 40 °C [10].

2.2. Standardization process

The *Phomopsis* extract was analyzed to meet the BPOM standard (BPOM Regulation No.32, 2019). The parameters analyzed were organoleptic, water and solvent content, microbial contamination, heavy metal contamination, and aflatoxin content [11]. Metabolite content was analyzed using LC-MS in three batches of extract production.

2.3. Molecular docking (MD)

Molecular docking was carried out using AutoDock Vina, and the main compound found in the extract was predicted for its anti-breast cancer potential using MD by attaching it to ERα and TK1. The 2D structure of the target compound was obtained from PubChem and converted into a 3D structure in the PDB format. The main compound from the *Phomopsis* extract acted as the test ligand. The ligand optimization results were saved in the PDBQT format.

The 3D structures of the protein targets (ER and TK1) in PDB format were obtained from the Protein Data Bank (PDB) database (http://www.rscb.org/pdb). The docking results were scored and the analysis

was carried out by looking at the best affinity energy (the most negative ΔG value), binding site similarity (BSS), hydrogen bonds, and hydrophobic interactions between the test ligand and the receptor/protein target [10].

3. Results and discussion

Phomopsis extract was successfully produced in three batches. Organoleptic analysis showed that the *Phomopsis* extract had a thick liquid appearance, with a characteristic odor of endophytic fungi, light brown color, and density of 0.941 g/mL (Figure 1c). The organoleptic results were consistent across all three batches.

The standardization results showed that the *Phomopsis* extract produced met the quality and safety standards according to BPOM (Table 1). The water content of the extract was 4.09%, and that of the BPOM standard was <10%. This indicates that the extract is safe for long-term storage. As for the solvent content, because the solvent used in the extraction process is ethyl acetate, which is not the solvent recommended by BPOM, it must be ensured that all the solvent has been removed in the final extraction results [11]. The results of this analysis showed that ethyl acetate was not detected in the *Phomopsis* extracts.

Some microbial and heavy metal contaminants were detected in the extract, but this value was still safe because it was below the BPOM threshold value. Meanwhile, for aflatoxin content, the results showed that all types of aflatoxin were not detected in the extract. Extracts that contain aflatoxins, microbes, and heavy metals that exceed standards can cause problems when they enter the body [12]. With the standardization results obtained, *the Phomopsis* extract can be declared safe for consumption.

Table 1. Standardization of the *Phomopsis* extract.

No	Parameter	Standard [15]	Result
1	Water content	≤ 10 %	4.09 %
2	Ethyl acetate solvent content	< 4 mg/L	Not detected
Microbial contamination			
3	Total plate count (TPC)	≤ 10^5 colony/g	≤ 10 colony/g
4	Yeast and mold	≤ 10^3 colony/g	≤ 10 colony/g
5	*Escherichia coli*	≤ 10 colony/g	<10 colony/g
6	*Enterobacteriaceae*	≤ 10^3 colony/g	10-100 colony/g
7	*Clostridium* sp	negative/g	negative/g
8	*Salmonella* sp	negative/g	negative/g
9	*Shigella* sp	negative/g	negative/g
Aflatoxin content			
10	Aflatoxin B1	≤ 20 µg/kg	Not detected
11	Aflatoxin B2	≤ 20 µg/kg	Not detected
12	Aflatoxin G1	≤ 20 µg/kg	Not detected
13	Aflatoxin G2	≤ 20 µg/kg	Not detected
14	Total Aflatoxin	≤ 20 µg/kg	Not detected
Heavy metal contamination			
15	Arsenic (As)	≤ 5 mg/kg	< 0.01 mg/kg
16	Mercury (Hg)	≤ 0.5 mg/kg	< 0.1 mg/kg
17	Cadmium (Cd)	≤ 0.3 mg/kg	< 0.004 mg/kg
18	Lead (Pb)	≤ 10 mg/kg	< 0.1 mg/kg

(a) (b) (c)

Figure 1. *Phomopsis* fungi on plate (a), cultivated in YMB (b), and the extract (c).

LC-MS analysis showed that there were 44 chemical compounds in the extract, the most abundant compound was 7-hydroxycoumarin [10]. These results are consistent with those of the extracts produced in the three batches, with an average relative abundance of 7-hydroxycoumarin of 50.5 % (Table 2). The consistency of the main compound content indicates standardization of the extract production process.

Table 2. Stability of the main compound content in three batches of extract production.

Compound	Formula	Batch	RT (min)	Area	Relative abundance (%)
7-hydroxycoumarin	$C_9 H_6 O_3$	1	11.989	4.9E+10	52.93
		2	11.998	3.9E+10	45.31
		3	11.990	4.9E+10	53.18
		Average ± standar deviation			**50.5 ±5.1**

An in silico study with MD showed that the main compound of the extract (7- hydroxycoumarin) could bind to RE and TK1, but the binding site similarity (BSS) values were not too large (41.18% and 52.38%). The hydrophobic interactions between 7-hydroxycoumarin and ERα were weaker than those between the native and comparative ligands. The affinity energy of this bond also had a lower negative value than those of the native and comparative ligands (Table 3). This indicated that 7-hydroxycoumarin does not have significant potential as an anti-breast cancer agent. However, it should be understood that in this *Phomopsis* extract, there are 43 other compounds, some of which have anti-breast cancer activity, based on previous studies.

Table 3. Molecular docking of 7-hydroxycoumarin with ERα and TK1.

Compound	Affinity energy (kcal/mol)	Inhibition constants (µm)	BSS (%)	Hydrogen bond	Hydrophobic interaction
Docking with **ERα**:					
• *Native ligand***:** 4-hydroxytamoxifen	-9.9	0.054		2	18
• *Comparatif ligand:* Raloxifen	-10.6	0.017		3	12
• 7-hydroxycoumarin	-6.7	12.118	41.18	3	7
Docking with **TK1**:					
• *Native Ligand:* p1-(5'-adenosil)p4-(5'(2'-deoksi-timidil))tetrafosfat	-12.1	0.0013		11	10
• *Compare Ligand*: Thymidine triphosphate	-11.4	0.0043	100	11	11
• 7-hydroxycoumarin	-6.8	10.234	52.38		11

Our previous *in silico* study with HER2 showed that some compounds in *Phomopsis* extract have a negative affinity energy around -9.3 and -9.4 kcal/mol. These results were better than trastuzumab (-7.5 kcal/mol), which is a widely used breast cancer drug [10]. These compounds were chalcone, 4-methoxy chalcone, diisoprenylxanthone, and 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione. Unfortunately, the relative abundance of these compounds in the extract was less than 1 % (each compound). This will be the next homework to analyze other compounds in the *Phomopsis* extract and their interactions with other proteins involved in the occurrence and treatment of breast cancer.

4. Conclusions

The water and solvent content, microbial, heavy metal, and aflatoxin contamination in the *Phomopsis* extract met BPOM requirements. The organoleptic results and metabolite contents of the *Phomopsis* extract were standardized. The metabolite content of the *Phomopsis* extract was consistent in the three batches of production, with the highest relative abundance being 7-hydroxycoumarin. *In silico* studies with molecular docking show that the binding of 7-hydroxycoumarin to estrogen receptor alpha (ERα) and thymidine kinase 1 (TK1) is not as good as the native and comparative ligand, because the affinity energy values are lower (-6.7 and -6.8 kcal/mol).

Acknowledgment

This study is part of a doctoral study supported by Universias Indonesia, IPB University, National Research and Innovation Agency (BRIN), The Center for Higher Education Fund - Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia, and The Indonesia Endowment Funds for Education.

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