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Metabolomic study and *in silico* approach of DLBS1442 as progesterone receptor agonist

Natalia Windari Rahardjo[1,2], Emanuel Dani Ramdani[1], Raymond Rubianto Tjandrawinata[1,2*], Yanti[2]

[1]Dexa Laboratories of Biomolecular Sciences, Cikarang 17550, West Java, Indonesia.
[2]Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta 12930, Indonesia.

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ABSTRACT

Endometriosis prevalence has been known to be quite high among women of reproductive age and with pelvic pain and/or infertility. The reason is that the estrogen level in the eutopic endometrium of women with endometriosis is higher than in normal endometrium which may possibly be caused by the lack of interaction between progesterone and progesterone receptor (PR). Dexa Laboratories of Biomolecular Sciences (DLBS) has developed DLBS1442, a bioactive fraction from *Phaleria macrocarpa* (Scheff) Boerl fruit, which has been found to be potential to treat symptoms of primary dysmenorrhea and alleviate endometriosis. Therefore, the identification of DLBS1442 active compounds which act as a PR agonist was necessary. Identification was performed using metabolomics study which resulted in 14 compounds. Crystal structure of the PR with asoprisnil as the reference was obtained from PDB (4A2J). Virtual screening validation process was performed using Protein-Ligand ANT System (PLANTS) and Python-based Protein-Ligand Interaction Fingerprinting (PyPLIF). According to the virtual screening protocol validation, the highest Enrichment Factor (EF) 1% value was obtained with hydrogen interaction with GLN725 and ARG766 residue. Virtual screening of the DLBS1442 metabolomics study showed that only glyceryl pentacosanoate exhibited a lower Chem Piecewise Linear Potential (ChemPLP) than the *cutoff*. This compound might have a role as a PR agonist which supported the previous findings of DLBS1442 to alleviate endometriosis. However, this finding requires further *in vitro* and/or *in vivo* study to ensure the agonist activity of glyceryl pentacosanoate as a DLBS1442 active compound.

INTRODUCTION

Endometriosis has been known as the most frequent cause of pelvic pain in women during reproductive years. Endometriosis is a non-life threatening condition in which tissue that normally lines a woman's uterus grows in other parts of the body, particularly on peritoneal tissues, bladder, ovaries, fallopian tubes, rectum, and other pelvic tissues. The prevalence of endometriosis is ~10%–15% among women of reproductive age (Crosignani *et al*., 2006; Mao and Anastasi, 2010; Vigano *et al*., 2004;) and up to 35%–50% among women with pelvic pain and/or infertility (Treloar *et al*., 2010). When endometriosis occurs, the eutopic endometrium experiences subtle abnormalities including biochemical reactions that increased the production of estrogen, prostaglandins, cytokines, and metalloproteinases (Bulun, 2009; Tjandrawinata and Rouli, 2017). These biological reactions resulted in pelvic pain, chronic pain, and fatigue which could lead to infertility.

In normal endometrium, progesterone exerts an antiestrogenic effect, in part by inducing 17β-hydroxysteroid dehydrogenase 2 (HSD17β2) which catalyzes the conversion of biologically potent estradiol to much less estrogenic estrone (Bulun *et al*., 2006). Progesterone acts on progesterone receptors (PRs) in endometrial stromal cells to increase the formation of retinoic acid as a paracrine factor, which induces HSD17β2 expression in endometrial epithelial cells. On the other hand, the estrogen level in the eutopic endometrium in women with endometriosis is higher than in normal endometrium, which may possibly be caused by the lack of interaction between progesterone and PR. As previously stated by Bulun *et al*. (2006), the increase of estrogen level in endometrial tissue is caused by progesterone resistance. Low level

*Corresponding Author
Raymond Rubianto Tjandrawinata, Dexa Laboratories of Biomolecular Sciences, Cikarang 17550, West Java, Indonesia.
E-mail: raymond @ dexa-medica.com

of progesterone is relatively related to prolonged release of estrogen and disordered proliferative endometrium. Prolonged estrogenic stimulation causes the endometrial glands to continue proliferating, becoming larger and more complex (Owings and Quick, 2014).

Selective PR modulator, such as asoprisnil, is a new class of PR ligands which have commonly been used in the studies of gynecological therapies such as uterine fibroids and endometriosis. This ligand can exert agonist and antagonist or mixed effects on various progesterone target tissues *in vivo* upon PR binding (Madauss *et al*., 2007). Asoprisnil leads to a less transcriptional activation than progestin and less transcriptional repression than antiprogestin. It also exhibits partial agonist/antagonist and tissue-selective effects in animals and humans. Although asoprisnil exhibits the potential to provide beneficial effects of progestins and antiprogestins, in this study, we focused on its agonist conformation as a model for molecular docking.

The development and use of the natural product have gained tremendous attention as a treatment of various disorders. Dexa Laboratories of Biomolecular Sciences (DLBS) has studied various herbals that exhibit potential effects on several diseases/disorders. For example is DLBS1425, a *Phaleria macrocarpa* extract which have antiproliferative and proapoptosis effects via eicosanoid pathway and downregulation of PI3K/AKT (Phosphatidylinositol-3-kinase/Protein kinase B) pathway (Tandrasasmita *et al*., 2010; Tjandrawinata *et al*., 2010). Other bioactive fraction from *P. macrocarpa* which called Proliverenol also has the potential ability in protecting cells from ethanol-induced hepatotoxicity (Berlian and Tandrasasmita, 2016). *In vivo* study by Tjandrawinata *et al*. (2015) presented that DLBS0533 which contains *P. macrocarpa* and *Nigella sativa* has an anti-inflammatory effect on mice model. An extract of pineapple (*Ananas comosus*) stem which named Tacorin and bioactive protein fraction isolated from *Channa striata* which called Striatin (DLBS0333) demonstrated acceleration of wound healing process by increasing cell proliferation (Rahayu *et al*., 2016; 2017). DLBS1442 is a proprietary and standardized semipolar bioactive fraction of *P. macrocarpa* (Scheff) Boerl fruit (Tjandrawinata *et al*., 2011). Previous clinical study indicated that DLBS1442 demonstrated its potential to treat symptoms of primary dysmenorrhea in premenstrual syndrome and alleviate endometriosis and/or adenomyosis related pain (Tjandrawinata *et al*., 2011, Wiweko *et al*., 2015). *In vitro* study by Tandrasasmita *et al*. (2015) reported that DLBS1442 significantly reduced the transcription level of the angiogenesis transcription factor, upregulated PR in RL95-2 cells, and induced cellular apoptosis in a dose-dependent manner. Therefore, DLBS1442 has been found to act as a potential agent to alleviate the symptoms of endometriosis via its antiangiogenic, anti-inflammatory, and proapoptotic activity. However, according to several activity studies of DLBS1442, the molecular mechanism of the active compounds that specifically play a role in generating its pharmacological effects has not yet been known.

In the present study, we investigated the bioactive compounds of DLBS1442 that may act as a PR agonist and evaluated its molecular activity. Our approach is to find the compound database of DLBS1442 and perform virtual screening. Database used in this study was obtained by the metabolomics approach. We hypothesized that the compound database of DLBS1442 could direct us to the compound that may possess an activity as a PR agonist.

MATERIALS AND METHODS

Preparation of receptor for virtual screening

X-ray crystal structure of the PR in complex with asoprisnil (PDB ID: 4A2J) was obtained from Protein Data Bank at www.rcsb.org (Lusher *et al*., 2012). X-ray crystal structure of the PR was separated from their bound ligand using Structure PrOtonation and REcognition System (SPORES) v1.3—mode splitpdb (Brink and Exner, 2009) and thus mol2 structures of 4A2J and asoprisnil were obtained. The crystal structure consists of two chains (A and B) where chain A was used. The binding site coordinates and the gridbox sizes were calculated based on binding site coordinates of asoprisnil in 4A2J using Protein-Ligand ANT Systems (PLANTS) v1.2—mode bind (Korb, 2009). The method also produced active site regions and active site amino acid residues as PLANTSactiveSite.mol2 and PLANTSactiveSiteResidues.mol2, respectively.

Comparing the binding pose of asoprisnil in X-ray crystal structure

Asoprisnil was re-docked to the crystal structure using PLANTS1.2_64bit—mode screen for 100 replicates. Cocrystal pose and re-dock pose of asoprisnil were superimposed in Pymol v2.10. Root mean square deviation (RMSD) of asoprisnil was calculated using command *rms_cur*. The visualization and RMSD calculation were performed in Pymol v2.10.

Decoy set and ligand set preparation

Decoy set (15,650) and ligand set (293) were obtained from DUD-E (dude.docking.org) in *.ism format (Mysinger *et al*., 2012). These compounds were converted into *.smi format then conditioned at pH 7.4. All files were converted into 3D (*.mol2) using Open-Babel v2.31 (O'Boyle *et al*., 2011). Decoys and ligands compound in *.mol2 were then processed using obconformer to get the best pose of each compound based on the Monte Carlo search. The *mol2 file was further processed using SPORES v1.3—mode reprot.

Virtual screening validation of 4A2J

Virtual screening was performed using PLANTS v1.2—*mode screen* with similar parameters used for redocking. For each compound, 50 binding poses were generated with triplicates replications. Other filtration was performed using Python-based Protein-Ligand Interaction Fingerprinting (PyPLIF) (Radifar *et al*., 2013), v0.1.1, which resulted in the *Tanimoto Coefficient* (TcPlif) score for each compound. Analysis result using Pylif v0.1.1 was then followed by Enrichment Factor (EF)1% rescoring based on the Chem Piecewise Linear Potential (ChemPLP) score from the best TcPlif value. The importance of hydrogen interaction of the ligand with GLN725 and ARG766 was also used as filtering system. Filtering was performed on compounds that exhibited hydrogen interaction with GLN725 and ARG766. EF1% was calculated based on the best ChemPLP score and TcPlif value.

Metabolomic study of DLBS1442

DLBS1442 was obtained from DLBS (Cikarang, West Java, Indonesia). Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) analysis was performed using Agilent

1290 Infinity II LC with 6545 quadrupole-time of flight (QTOF) MS Detector. Separation was performed using XTerra MS C18 with 3.0 × 50 mm, 3.5 µm column. Acetonitrile (A) and ultrapure water (B) were used as the solvent. At a flow rate of 0.6 ml/minute, a gradient chromatographic system was performed using 2% solvent A into 100% solvent A for 11 minutes, followed by an equilibration of 2% solvent A over the next 4 minutes. MassHunter Workstation (B.06.01) was used as the processing software. The acquisition of MS/MS detector was performed at positive ion mode using Dual Agilent Jet Stream Electrospray ionization as an ion source.

LC-MS/MS result was processed using MZmine-2.32 (Pluskal *et al*., 2010), and the *m/z* of parent ion and fragmented ion was then collected. For compound prediction, we used METLIN at www.metlin.scripps.edu (Guijas *et al*., 2018). The *simple search* mode was used for early screening using the *m/z* value of the precursor ion. Predicted compounds from simple search were then continued with *fragment similarity search* using the *m/z* value of the precursor and fragment ions. Other references such as journals related to the metabolite compounds of DLBS1442 were also used in this metabolomics study.

Virtual screening of metabolomic study result

The structure of compounds from metabolomics study was obtained from PubChem (https://www.ncbi.nlm.nih.gov/pccompound) with *.sdf format, while the structure of new compound which was unavailable was generated using ChemDraw Ultra 12.0.2.1076 and saved as *.sdf format as well. Virtual screening was performed using PLANTS v1.2—*mode screen* using the validated protocol.

RESULTS AND DISCUSSION

Virtual screening protocol validation

One commonly used method for validating docking program is pose selection whereby docking programs are used to re-dock into the target's active site, compound with a known conformation and orientation, typically from co-crystal structure. In this study, the cocrystallized structure of PR in complex with asoprisnil (PDB ID: 4A2J) was used. The docking results showed that the binding conformation of the redocked asoprisnil reproduced the RMSD of less than 2.0 Å, which was ranged from 0.962 to 0.973, as shown in Figure 1. Moreover, Figure 1 showed that the shifting of redocking results of asoprisnil (yellow) was insignificantly different compared to the cocrystal pose (turquoise blue). According to Hevener *et al*. (2009), programs that are able to obtain an RMSD value of lower than 2.0 Å were considered to exhibit a successful performance.

Virtual screening protocol validation for a PR agonist bind to asoprisnil crystalline structure was performed retrospectively. DUD/DUD-E is an example of a benchmark designed for validating virtual screening protocols and publicly available virtual screening test database (Mysinger *et al*., 2012). 15,650 decoys and 293 ligands in *.mol2 files from DUD-E were docked, and the protocol was then assessed for its ability to distinguish ligand from decoys. The assessed validation parameter was EF1% that is defined as the ratio of number of active compounds retrieved relative to the number of database molecules tested. Higher EF1% has been known to represent better protocol for ligand recognition because from the first 1% of the sorted

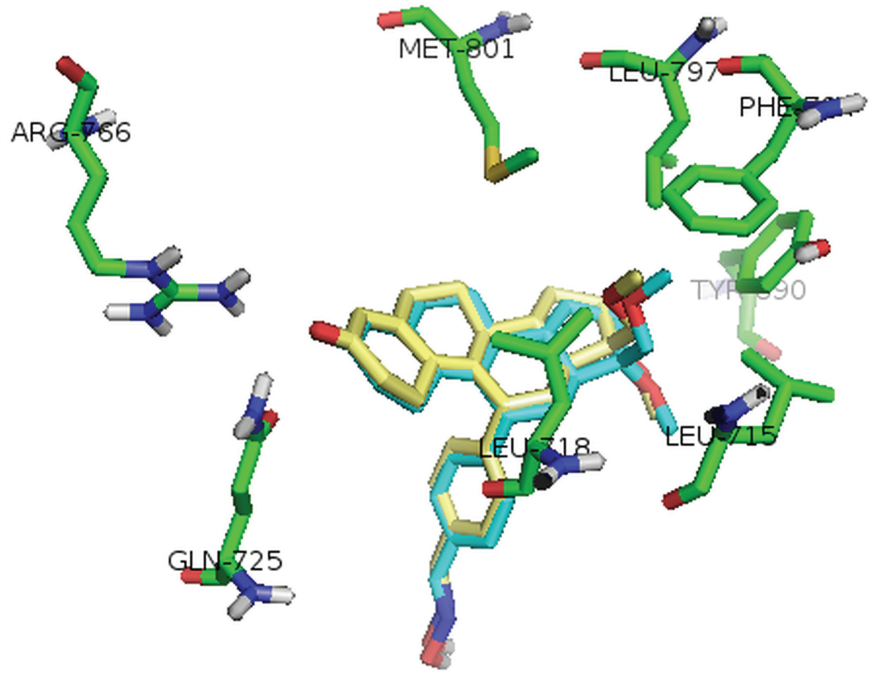

Figure 1. The superimposition between the docked conformation (yellow) and the cocrystal structure (turquoise blue) of the progesterone receptor-asoprisnil complex (4A2J).

database, the protocol was able to recognize ligands and exhibit higher ranks than decoys (Megantara *et al*., 2016).

Virtual screening protocol validation was performed using PLANTS (Korb, 2009). PLANTS has commonly been used for virtual screening as studied by Istyastono *et al*. (2015). All decoys and ligands were converted into *smile* format and prepared using Open Babel to adjust hydrogen atom into pH 7.4 that is similar to body's condition. The *smile* format was then converted into three-dimension structure *mol2* format using the same program, which could also be read by PLANTS. The *mol2* format was processed using obconformer to get the best pose of each compound.

mol2 files of decoys and ligands were prepared using SPORES that performed structure recognition, and thus the three-dimension structures were adjusted to the algorithm suitable for PLANTS. The protonation by SPORES can either be done by adding the missing hydrogen atoms or as a complete reprotonation. List of amino acid residues which play a role in binding site was obtained using PLANTS. According to Lusher *et al*. (2012), oxosteroids, which include progesterone and asoprisnil, exhibit classic interactions to GLN725 and ARG766 with attachment to the hydrophobic pocket which consists of LEU715, LEU718, PHE794, LEU797, MET801, and TYR890.

Two scoring functions that were used for virtual screening validation consisted of ChemPLP and Tanimoto coefficient PLIF score (TcPLIF). PLANTS ChemPLP is an empirical scoring function that computes the fitness of protein−ligand binding by summing up the contributions of a number of individual terms, each represents an important energetic factor in protein−ligand binding (Liu *et al*., 2015). As a complementary method to ligand docking, TcPLIF can be applied to quantify the similarity of the predicted binding poses to a reference binding pose (Rácz *et al*., 2018).

Four protocols were used in the validation process. EF1% results from the protocols were tabulated in Table 1.

According to the results in Table 1, the best EF1% value, which is 7.16 was obtained on the protocol with hydrogen interaction with GLN725 and ARG766 residue and continued by rescoring based on ChemPLP score. This result was in accordance with the previous experiment by Williams and Sigler (1998) and Lusher *et al*. (2012) which found that hydrogen interaction with GLN725 and ARG766 is considered to be a vital interaction for both agonist and antagonist of PR functions.

Based on the best virtual screening protocol, the ChemPLP *cutoff* was −63.47. A compound could possibly be a ligand that acts as a PR agonist if it exhibits hydrogen interaction with GLN725 and ARG766 with the ChemPLP score −63.47.

Metabolomic study of DLBS1442

Metabolomic study was performed using LC-MS/MS with setting parameters as stated in the method. The gradient composition of the mobile phase consisted of 2% acetonitrile into 100% acetonitrile for 11 minutes, then continued with 4 minutes of 2% acetonitrile for equilibration. The low percentage of organic solvent at the beginning of analysis was intended to retain the DLBS1442 sample, so the compounds contained in DLBS1442 extract could be eluted gradually.

Table 1. EF1% value for each virtual screening protocol.

Protocol	EF1% based on	
	ChemPLP score	TcPlif
Without GLN725 and ARG766 interaction	3.41	4.77
With GLN725 and ARG766 interaction	7.16	5.46

LC-MS/MS analysis provides information about precursor ions and fragment ions in positive ion mode $[M+H]^+$. METLIN database was used to predict the structure of compounds from LC-MS/MS analysis results. *Fragment similarity search* in METLIN was used for compound identification approach.

Chromatogram of DLBS1442 showed that the highest peak gave a retention time of approximately 3.1 minutes. *m/z* value of the peak at retention time 3.04 minutes was 261.0762 and 423.1292. After mass fragmentation, precursor with *m/z* value of 423.1289 exhibited *m/z* value for fragmented ions of 261.0756, 167.0341, 121.0287, and 85.0286. However, identification in the METLIN database using both *fragment similarity search* and *MS/MS spectrum match search* did not gave any information about the *m/z* values. Literature study was also performed during the present metabolomics study. A study by Oshimi *et al*. (2008) stated that phalerin is one of the major compounds found in *P. macrocarpa* fruits and is believed to possess medicinal effects. Based on its structure, phalerin is a benzophenone glucoside which has been identified as *2,4',6-trihydroxy-4-methoxybenzophenone-2-O-glucoside*. After comparing MS data of DLBS1442 and phalerin standard, it was found that *m/z* value of 261.0761 and 423.1287 was also found in phalerin standard with similar retention time at 3.03 minutes (Fig. 2). Precursor ion with *m/z* value 423.1287 in phalerin was also fragmented into 261.0756, 167.0342, 121.0287, and 85.0286. This data suggests that precursor with *m/z* value 423.1289 at retention time 3.04 minutes in DLBS1442 might be phalerin.

Other predicted compounds from DLBS1442 were tabulated in Table 2. Due to the limitation of the database used, other references such as journals related to the metabolite compounds of DLBS1442 were also used for the metabolomics study. Ramdani *et al*. (2017) have identified several compounds in DLBS1442, which include glyceryl pentacosanoate, *1,7-dihydroxy-3,6-dimethoxyxanthone*, *1,6,7-trihydroxy-3-methoxyxanthone*, *2,4',6-trihydroxy-4-methoxybenzophenone-2-O-glucoside*, *β-Sitosterol*, Coumarin, and *2,3-dihydroxybenzoic acid*.

Activity prediction of metabolomic study result as PR agonist

PR agonist activity from metabolomics study was predicted using virtual screening protocol which has previously been validated. Sample preparation, molecular docking parameters, and data processing were performed similar to the virtual screening protocol validation.

All identified compounds of DLBS1442 were used for activity prediction. Virtual screening result showed that there is only one compound that possesses ChemPLP ≤ −63.47 (Table 3). The lowest ChemPLP was obtained in glyceryl pentacosanoate with a ChemPLP score of −87.29. This compound is a small molecule that exhibits hydrogen interaction with GLN725 at 1.7 Å and ARG766 at 2.0 Å. These distances were closer compared to the asoprisnil 2.2 Å and 2.4 Å with GLN725 and ARG766, respectively (Fig. 3A and B). This closer distance for interaction with GLN725 and ARG766 could be supported with the low ChemPLP which indicates that glyceryl pentacosanoate could

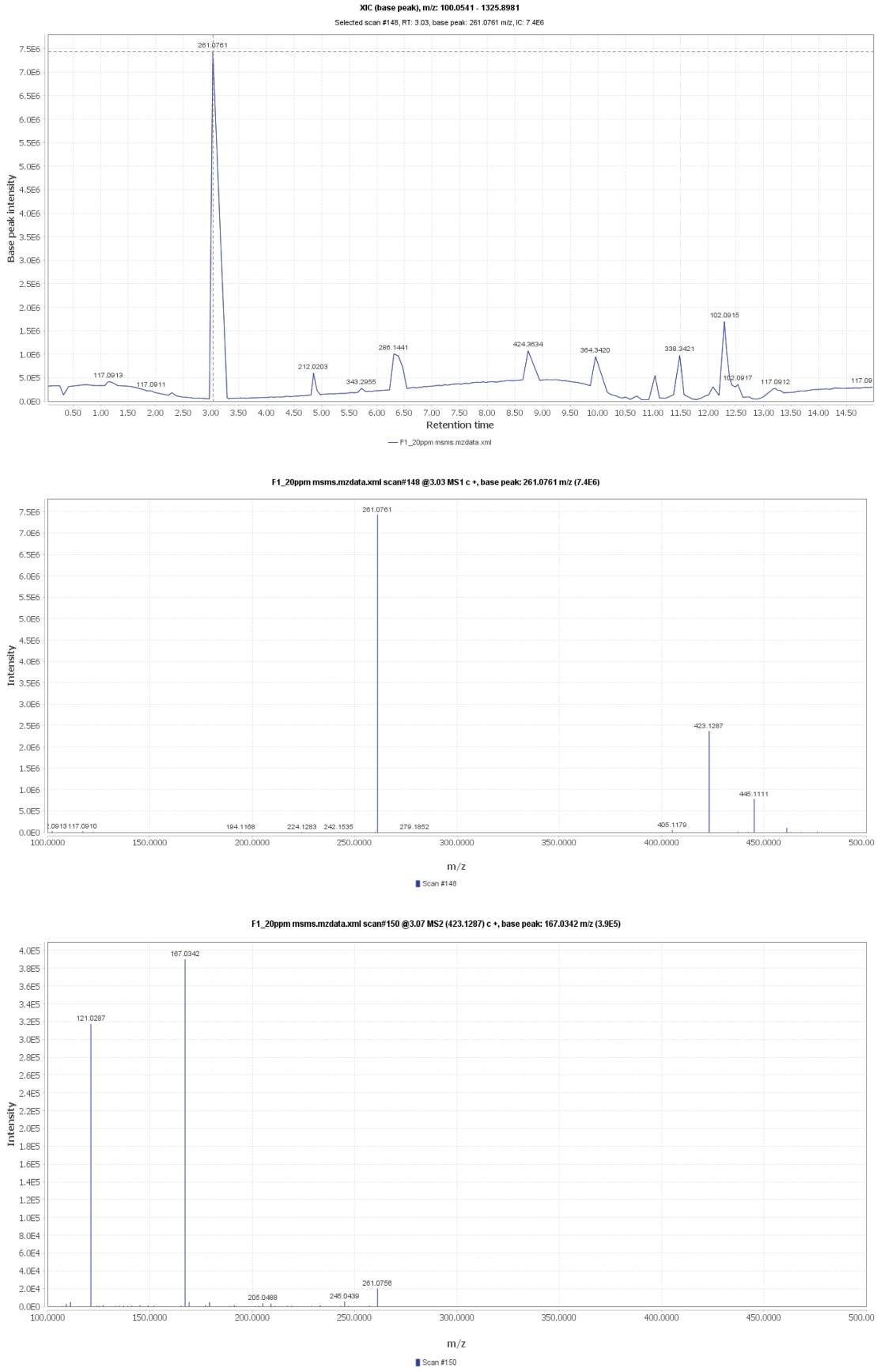

Figure 2. Chromatogram of phalerin (1), MS (2), and MS/MS (3).

Table 2. Compounds identification from DLBS1442 based on metabolomic study.

No.	*m/z*	Retention time (minutes)	Compound	Base peak intensity	Group
1	289.0712	6.25	1,7-dihydroxy-3,6-dimethoxyxanthone	6.5×10^4	Xanthone
2	275.0554	5.54	1,6,7-trihydroxy-3-methoxyxanthone	2.7×10^5	Xanthone
3	268.1049	1.99	Vidarabine	7.0×10^5	Glycoside
4	423.1042	2.95	Mangiferin	1.5×10^6	Xanthone
5	261.0762	3.06	2,3,4'-trihydroxy-4-methoxybenzophenone	4.8×10^6	Benzophenone
6	423.1289	3.08	2,4',6-trihydroxy-4-methoxybenzophenone-2-O-glucoside (Phalerin)	1.5×10^6	Glycoside
7	447.1288	3.94	Glycitin	3.4×10^4	Flavonoid
8	342.2067	7.83	Gangetin	1.6×10^5	Flavonoid
9	338.3421	11.44	13Z-Docosenamide	1.4×10^6	Fatty acid
10	457.0969	2.92	Glyceryl pentacosanoate	5.1 x 10^3	Ester
11	465.1388	3.75	2,4',6-trihydroxy-4-methoxy-6"-acetylbenzophenone-2-O-b-D-glucoside (Mahkoside B)	6.5×10^3	Glycoside
12	N/A	N/A	Coumarin	N/A	Benzopyrone
13	N/A	N/A	2,3-dihydroxybenzoic acid	N/A	Carboxylic acid
14	N/A	N/A	β-Sitosterol	N/A	Phytosterol

Table 3. Virtual screening result from the metabolomic study of DLBS1442 compounds.

No.	Compound name	ChemPLP
1	Glyceryl Pentacosanoate	−87.29
2	Mangiferin	−38.27
3	Glycitin	−23.01

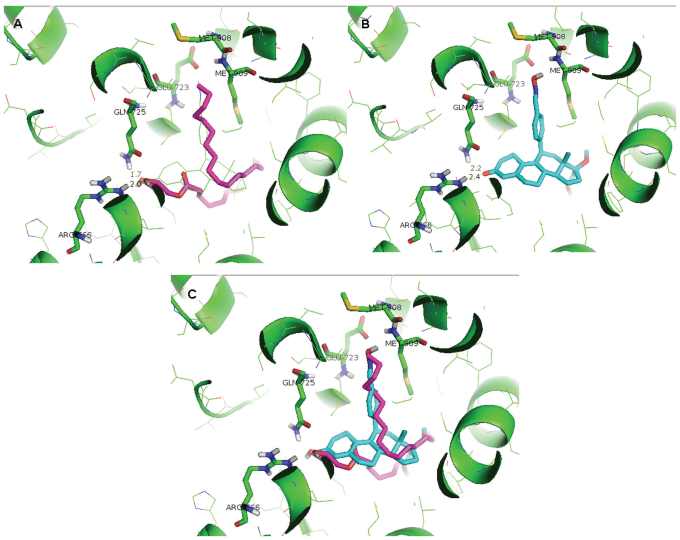

Figure 3. Interaction of GLN725 and ARG766 with (A) glyceryl pentacosanoate and (B) asoprisnil. (C) Overlay position of glyceryl pentacosanoate and asoprisnil.

spontaneously interact with the PR. Figure 3C shows the best pose of glyceryl pentacosanoate as a PR agonist compared to asoprisnil.

In silico study of DLBS1442 found that glyceryl pentacosanoate might have a role as a PR agonist. This result supported the previous study by Tandrasasmita *et al*. (2015) which found that DLBS1442 could also increase the expression of the PR. This finding shows the agonist-like activity of DLBS1442 to the PR, thus allowing better understanding of its molecular activity as anti-endometriosis in women. However, this finding requires further *in vitro* and *in vivo* approach to ensure the agonist activity of glyceryl pentacosanoate as the active compound of DLBS1442.

CONCLUSION

The virtual screening protocol for PR agonist has retrospectively been validated using PLANTS, PyPLIF, and DUD-E database. On the other hand, the metabolomics study of DLBS1442 also leads us to the specific compounds which might have a role for its activity. Virtual screening of the metabolomics study of DLBS1442 showed that only glyceryl pentacosanoate exhibits ChemPLP ≤ -63.47 and hydrogen interaction with GLN725 and ARG766. This indicates that glyceryl pentacosanoate could possibly play an important role as a PR agonist. Further approach by *in vitro* and *in vivo* studies would provide great support to prove its medical benefit for human life.

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CONFLICT OF INTEREST

Authors declare that there are no conflicts of interest.

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