Philippine Journal of Science
151 (5): 1771-1791, October 2022
ISSN 0031 - 7683
Date Received: 02 Feb 2022

Screening *Rafflesia* and *Sapria* Metabolites Using a Bioinformatics Approach to Assess Their Potential as Drugs

Adhityo Wicaksono[1]*, **Reza Raihandhany**[2], **Tresa Variyani Zen**[3], **Jaime A. Teixeira da Silva**[4], **Aggy Agatha**[1], **Ghea Putri Cristy**[1], **Arfan Tri Kusuma Ramadhan**[1†], and **Arli Aditya Parikesit**[5*]

[1]Division of Biotechnology, Generasi Biologi (Genbinesia) Foundation,
Jl. Swadaya Barat No. 4, Gresik 61171 East Java, Indonesia
[2]Division of Botany, Generasi Biologi (Genbinesia) Foundation,
Jl. Swadaya Barat No. 4, Gresik 61171 East Java, Indonesia
[3]Science Unit, Wildlife Conservation Society,
Jl. Malabar 1 No.11, Bogor, Jawa Barat 16128 Indonesia
[4]Ikenobe 3011-2, Kagawa-ken 761-0799 Japan
[5]Department of Bioinformatics, School of Life Sciences,
Indonesia International Institute of Life Sciences, Jl. Pulomas Barat No. Kav. 88,
East Jakarta 13210, Jakarta Capital Region, Indonesia

The Rafflesiaceae family consists of three genera of parasitic plants – *Rafflesia, Rhizanthes,* and *Sapria* – with purported ethnobotanical and ethnomedicinal properties. In this study, the inhibitory properties of 21 characterized metabolites associated with *Rafflesia* and *Sapria* were tested against eight proteins linked to human diseases – including seven pathogenic-associated HMGCR, VEGFR2, acetylcholinesterase, NMT, H1N1 neuraminidase, GSK3-β, and estrogen receptor α, and one plant-pathogenic associated *Colletotrichum* chitin deacetylase. Each metabolite was tested using drug-likeness screening, screening metabolite activity, and molecular docking to eight diseases and microbial physiological processes. Hydrogen bonds and hydrophobic interactions between metabolite ligands and protein residues were characterized. Molecular dynamics were also assessed to analyze the stability of the protein-ligand interaction. Our results indicate that the gallotannins and flavonol phenolics from *Rafflesia* and *Sapria* display high inhibitory potential against disease proteins. All metabolite-protein pairs displayed stable fluctuations. However, some compounds disobeyed LRO5 drug-likeness and displayed moderate bioavailability and synthetic accessibility, so an improved drug delivery method is required. All 21 metabolites are available in other popular edible plants (mainly tea and certain berries) and could be used to create artificially mixed metabolite-based medicine to prevent the exploitation and endangerment of wild *Rafflesia* and *Sapria* populations. Our activity likelihood screening and molecular docking data indicate that *Rafflesia* and *Sapria* metabolites possess considerable potential as anti-cholesterol, respiratory antiviral, wound-healing, and antifungal properties. To protect Rafflesiaceae plants in the wild, metabolites can be assessed from other plant sources and combined as an artificial herbal mix.

Keywords: alkaloids, ethnobotany, folk medicine, phenolic, Rafflesiaceae, secondary metabolite

*Corresponding author: adhityo.wicaksono@gmail.com
arli.parikesit@i3l.ac.id

†Deceased: 07 April 2022

INTRODUCTION

Members of the Rafflesiaceae family are well-known for their unique floral morphology and consist of holoparasitic endophytes with highly modified reproductive structures and an extremely reduced vegetative body (Nikolov *et al.* 2014). All three genera of the Rafflesiaceae (*Rhizanthes*, *Rafflesia*, and *Sapria*) do not possess any leaves, stems, or roots and are obligate parasites of the genus *Tetrastigma* (Vitaceae), on which they form an endophytic system inside the living bark of host plants (Meijer 1993). The most striking feature of the Rafflesiaceae is the large red flower produced by *Rafflesia* species, making them an iconic attraction for ecotourism in Indonesia and other countries in Southeast Asia such as Malaysia, the Philippines, and Thailand. Several challenges in the *ex situ* conservation of members of this family remain since natural populations are rare in nature and difficult to propagate (Nikolov *et al.* 2014; Mursidawati and Irawati 2017).

Rafflesia possesses the largest known individual flowers among the three genera and has been recognized for its ethnomedicinal properties in some regions of Southeast Asia. In Malaysia and Indonesia, *Rafflesia* buds are used locally as a postpartum restorative tonic, and the commonly used species are *Rafflesia hasseltii* Suringar (Wiart *et al.* 2014) and *Ra. zollingeriana* Koord. (Zaman 2009). *Rafflesia* buds are also utilized by individuals in the Malayan Peninsula to stop internal bleeding and to shrink the vaginal shaft following childbirth (Nais 2001). However, it is unknown which species are specifically used (Mat-Salleh 1991; Nais 2001). Additionally, *Rafflesia* is also used as an energy drink for men (Kanchanapoom *et al.* 2007; Refaei *et al.* 2011) to cure fever and backache (Ismail 1988; Meijer and Elliott 1990; Hikmat 2006), as well as a supplement for women's fertility (Zuhud *et al.* 1999). Similarly, *Rhizanthes* is also known for its ethnomedicinal properties in Indonesia to cure several diseases such as hemorrhages, back pain, stomachache, and hematuria (Quattrocchi 2012; Syaifuddin *et al.* 2018) – specifically using *Rhizanthes lowii* (Becc.) Harms, which is known locally as "ulur-ulur" (Syaifuddin *et al.* 2018; Wicaksono *et al.* 2021). None of these ethnomedicinal properties have been tested in clinical trials on animals or humans.

In addition, until now, the secondary metabolites of *Rhizanthes* have not been identified, unlike *Rafflesia* and *Sapria*. A study carried out by Saleh *et al.* (2015) revealed that the crude ethanolic extract of *Rh. deceptor* Bänziger & B.Hansen flower buds contained secondary metabolites such as alkaloids, flavonoids, phenolics, and triterpenoids. Although these groups of metabolites were revealed, the precise identity of the chemical compounds remains unknown, as no additional studies have been conducted yet. Saleh *et al.* (2015) also tested the ethanolic extract of *Rh. deceptor* flower buds – revealing antioxidant and pesticidal properties – tested using 1,1-diphenyl-2-picrylhydrazyl or DPPH antioxidant test and the brine shrimp lethality test, respectively. In terms of *Sapria*, an ethnobotanical study carried out by Wangchuk *et al.* (2011) showed that the flowers of *Sapria himalayana* Griff. were claimed by locals to alleviate liver disorders and fever. Despite the generally wide distribution of the *Sapria* genus in Assam, south-central China, and Indo-China (Plants of The World Online 2017), no other ethnomedicinal uses of *Sapria* – other than the descriptions provided by Wangchuk *et al.* (2011) – are available.

Independent of the promising nature of the medicinal prospects of the Rafflesiaceae, some species are protected by law in Southeast Asian countries – especially in Indonesia, Malaysia, the Philippines, Thailand, and Vietnam. In Indonesia, *Rafflesia* species are considered an endangered genus, protected by Indonesian Law Year 1990 Number 5, which governs the conservation of biodiversity and their ecosystems (Indonesian Ministry of Foreign Affairs 1990). Endangered floras, according to the 2008 IUCN Red List, are protected by law in Malaysia – the 1972 Protection of Wildlife Act applies to Peninsular Malaysia (UN Environment Programme 2021) – whereas the 1997 Sabah Wildlife Conservation Enactment (Sabah Law Net 2010) and the 1998 Sarawak Wildlife Protection Ordinance (Sarawak Forestry 1998) apply to Sabah and Sarawak (Malaysia), respectively. In the Philippines, biodiversity is protected under 1995 Executive Order No. 247, Section 14 of the Wildlife Act (Republic Act No. 9147) (Official Gazette of the Government of Philippines 2001). In Thailand, three *Sapria* species became protected according to the Department of National Parks, Wildlife, and Plant Conservation (2017) – all of which are included in the 2001 IUCN Red List: *Sapria himalayana* Griff. is classified as vulnerable, whereas *Sapria poilanei* Gagnep. and *Sapria ram* Bänziger & B.Hansen are classified as endangered.

Even though select metabolites in *Rafflesia* and *Sapria* were described by Sofiyanti *et al.* (2008), Kanchanapoom *et al.* (2007), and Iwashina *et al.* (2020) (Appendix I Table 1), *in silico* approaches such as molecular docking and drug prediction using an online database (*e.g.* SwissADME) for the drug prediction test and PASS Online to test the potential bioactivity of a metabolite can be used as a non-destructive (because it relies exclusively on existing metabolite data), yet powerful analytical method to acquire preliminary data based on simulation as the first step to decide whether it can be processed for the next step: as a clinical trial (Wicaksono and Muttaqin 2020). Using available metabolites from the existing literature as ligands, combined with specific proteins from an online database, molecular docking can be performed to test the molecular interactions between ligands (metabolites) and

specific disease-related proteins in humans or microbes, thereby serving as a method for drug screening (Gschwend *et al.* 1996; Ferreira *et al.* 2015).

Despite the ethnomedicinal potential of Rafflesiaceae, the rarity of plants in this family stems from its complex ecology that is strictly dependent on a host plant. Rafflesiaceae species can only grow when their habitat meets the specific ecological requirements of the host plant, *Tetrastigma* (Wicaksono *et al.* 2016, 2021). Further requirements for the presence of Rafflesiaceae includes a viable population of carrion flies as pollinators, pollination efficiency (*i.e.* if pollen is transported efficiently from male to female flowers), flowering period (*i.e.* it will be problematic if male flowers bloom but there are no female flowers, and *vice versa*), seed dispersal and dormancy (*i.e.* if seeds are well-dispersed and maintain their viability throughout the process), and the chance of germination (especially if there are cases of agamospermy) (Bänziger 1991). If populations of these rare Rafflesiaceae species were to be harvested for ethnomedicine, there could be a rapid and irreversible decline in the wild if not counterbalanced by propagation studies (Wicaksono *et al.* 2016). Moreover, the first step to determining if metabolites possess drug-like properties is to confirm if the metabolites are truly endemic to the Rafflesiaceae.

The main objective of this study was to uncover the potential use of *Rafflesia* and *Sapria* metabolites for drug use. This was achieved by performing a drug-likeness and bioactivity test for each metabolite, simulations of molecular docking to test the binding affinity between these metabolites as ligands and targeted proteins, and molecular dynamics for a ligand-protein complex to check the protein's structural stability during that interaction. The focus was on proteins detected in previous studies related to ethnobotanical and pharmaceutical uses. These proteins were selected because they are associated with major diseases around the world (see details in the methods section).

After completing *in silico* testing, the metabolites' potential against standard drugs for the eight tested proteins was compared. Finally, to determine if these metabolites are specific to *Rafflesia* and *Sapria*, or to appreciate their availability more widely, the literature was assessed. The latter step served to understand if there were easy-to-find and easy-to-grow commercially available plants from which the same metabolites could be extracted, allowing them to be more easily obtained without harming endangered and protected *Rafflesia* and *Sapria* resources. For metabolites that are specific to *Rafflesia* and *Sapria*, a future biotechnological approach could employ genetically engineered microbes or plants that produce the designed drugs. In terms of the availability of metabolites, commercially grown plants are evidently a more sustainable solution than the use of rare and endangered *Rafflesia* and *Sapria*. Ultimately, since conservation is still the primary goal for these parasitic plant genera, this research aims to provide a multidisciplinary approach and perspectives that simultaneously accommodate future Rafflesiaceae studies in biotechnology, industry, and conservation.

MATERIALS AND METHODS

Metabolite Ligands

A total of 21 compounds were used (Figure 1), including alkaloids and phenolics (Sofiyanti *et al.* 2008), as well as gallotannins and other metabolites (Kanchanapoom *et al.* 2007; Iwashina *et al.* 2020). Almost all of the metabolites' Simplified Molecular Input Line Entry System (SMILES) codes and PDB files were obtained from the Human Metabolome Database (HMDB) (https://hmdb.ca/), except for the gallotannins from *Rafflesia kerrii* Meijer and the *S. himalayana* digalloyl, trigalloyl, and tetragalloyl glucose tannins. The structures of gallotannins that were not available in HMDB were redrawn based on descriptions by Kanchanapoom *et al.* (2007) using the SMILES online checker and SMILES converter in Cheminfo.org (http://www.cheminfo.org/flavor/malaria/Utilities/SMILES_generator___checker/index.html). All SMILES codes that were obtained were converted into PDB files at the CADD Group Chemoinformatics Tools and User Services (CACTUS) translator website (https://cactus.nci.nih.gov/translate). These files were used to screen the drug-likeness and drug bioactive potential of these metabolites. Furthermore, these metabolites were used as ligands in protein-ligand docking. All 21 metabolites and their IDs according to HMDB, ChemSpider, and PubChem CID, are listed in Appendix I Table 1.

Protein Macromolecule Targets

Eight proteins were tested against the 21 metabolites and compared to some control ligands, including natural ligands, if available. Recognized inhibitor drugs were also used for comparison (Figure 1). The tested proteins were acquired from the RCSB PDB database website (https://www.rcsb.org/) in PDB format. The proteins' active site residues were noted using BIOVIA Discovery Studio 2021 (v.21.1.0.20298). For protein PDB that had no available ligand, active site residues were predicted using 3DLigandSite (https://www.wass-michaelislab.org/3dlig) developed by the Wass-Michaelis Research Group of the University of Kent, UK (Wass *et al.* 2010) by submitting the protein sequence. The natural ligand substrates and inhibitor drug ligand PDB files that were used for comparison were browsed and downloaded from the

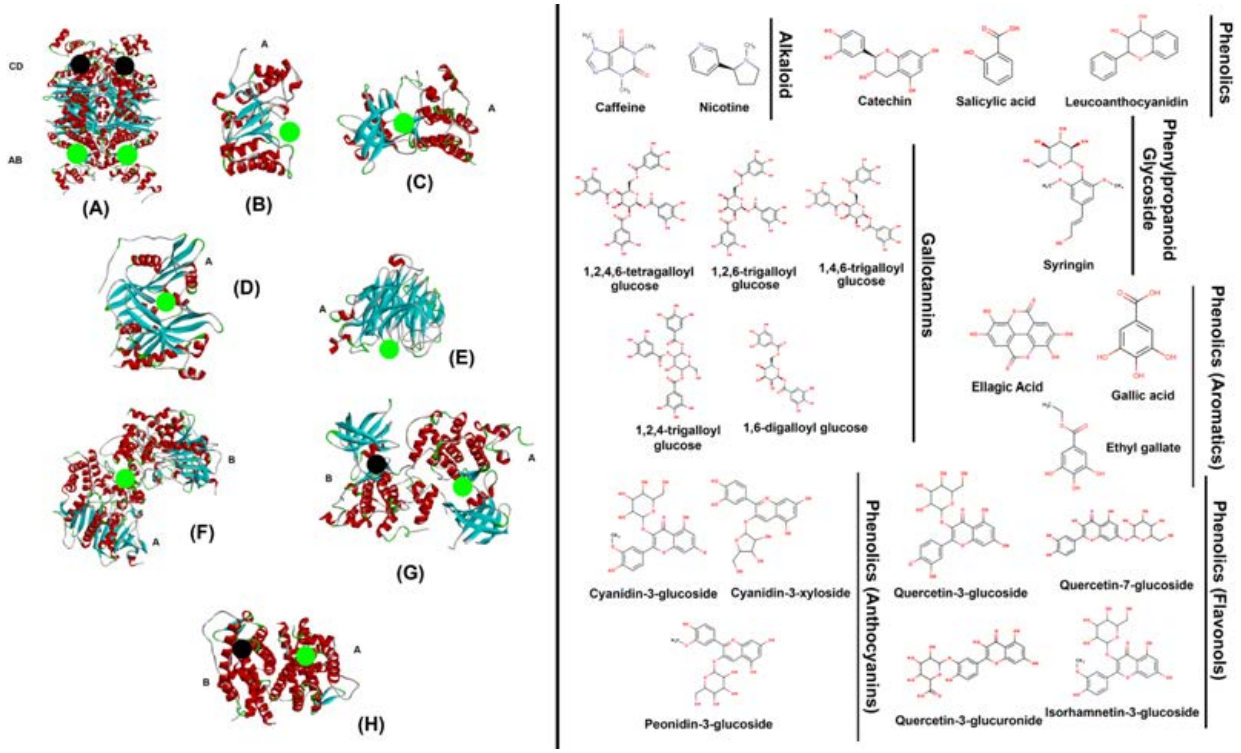

Figure 1. Proteins used in this study and their binding sites (left; black – alternative active site, and green – selected active site for docking) and the metabolite ligands (right). Proteins including 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (A), *Colletotrichum* chitin deacetylase (B), vascular endothelial growth factor receptor 2 (VEGFR2) (C), *Aspergillus fumigatus* N-myristoyl transferase (NMT) (D), influenza A virus neuraminidase single unit (E), acetylcholinesterase (F), glycogen synthase kinase 3-β (G), and estrogen receptor α (H). Small letters indicate the chains. The red area indicates the α-helix, whereas the blue indicates the β-sheet. Proteins viewed in BIOVIA Discovery Studio 2021 and ligands with SwissADME.

Human Metabolome Database web server (https://hmdb.ca/). The interactions of the tested proteins against these ligands served as the controls. The 21 tested metabolite ligands are supposed to have more negative binding affinity than the control ligands.

The first protein group covers internal pathological diseases. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) protein (PDB: 1DQ8), which is involved in the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevaldyl-CoA, is related to sterols and isoprenoid, so it is targeted in patients with cholesterol-related diseases (Istvan *et al.* 2000). This protein PDB in RCSB is attached to its natural ligand substrate 3-hydroxy-3-methylglutaryl (HMG) and coenzyme-A (CoA), and from all binding sites (two pairs of active sites) (Istvan *et al.* 2000), two active sites located in chains A and B were selected since these have the protein residues related to attachment listed for docking. Atorvastatin, which is an inhibitory cardiovascular disease drug to deal with abnormal lipid levels (Ye *et al.* 2015), was used as an additional control ligand.

The next protein that plays a role in breast cancer development, vascular endothelial growth factor receptor 2 (VEGFR2; PDB: 3VHE), attached to the inhibitor drug ligand pyrrolopyrimidine (Oguro *et al.* 2010). Breast cancer was selected as it is highly diagnosed in the world, surpassing lung cancer in 2020 according to GLOBOCAN (Sung *et al.* 2021). Many studies involving plant-based metabolites have targeted the inhibitory effect on VEGFR2 protein that triggers angiogenesis in cancer tissues (Seo *et al.* 2013; Wang *et al.* 2020). Along with pyrrolopyrimidine, an additional breast cancer therapeutic drug approved by the FDA in 2005 that targeted VEGFR2, sorafenib (Peng *et al.* 2017; Alam and Khan 2018; Wang *et al.* 2019), was also used as a comparative ligand. A protein that is normally targeted for patients with Alzheimer's disease as an inhibitory drug – acetylcholinesterase (AchE) – was selected (PDB 6O4W, chain A), as was the inhibitory drug ligand dopafenib (Gerlits *et al.* 2019).

To test for an aphrodisiac effect, metabolite ligands were targeted to estrogen receptor α (PDB: 1X7E, chain A), and as the control, the attached ligand molecule of [5-hydroxy-2-(4-hydroxyphenyl)-1-benzofuran-7-yl]acetonitrile (WAY-244) was used (Manas *et al.* 2004). A previous

study tested estrogen receptors with phytochemicals using molecular docking to test the potential of estrogen-mimicking compounds that might work as an aphrodisiac (Powers and Setzer 2015).

For wound healing activity, the protein glycogen synthase kinase 3-β (GSK3-*β*) (PDB: 1Q5K, chain A) with its attached inhibitor ligand, N-(4-methoxybenzyl)-n'-(5-nitro-1,3-thiazol-2-yl)urea (TMU) was subjected as the target (Bhat *et al.* 2003). In the wound-healing mechanism, the inhibitory action of GSK3-*β* enhance wound healing *via* regulation of the Wnt/*β*-catenin pathway (Zhang *et al.* 2008; Vidya *et al.* 2012).

For activity related to pathogens, fungal and viral proteins were used as targets. The fungus used in this study was *Aspergillus fumigatus*, a saprotrophic fungus that is abundant in the air, and its conidia pose a danger to immunocompromised patients, leading to brachiopulmonary aspergillosis (Latgé 1999). N-myristoyl transferase (NMT) is an enzyme that plays a role in fungal cell development by modifying lipids by transferring the 14-carbon saturated fatty acid myristate from myristoyl-CoA to the N-terminal glycine residue (Boutin 1997; Bhatnagar *et al.* 1998; Sogabe *et al.* 2002; Guerrero-Perilla *et al.* 2015) [PDB: 4CAW; attached with natural ligand Myristoyl-CoA and inhibitory drug ligand pyrazole sulfonamide (Fang *et al.* 2015)]. The inhibitory ligand, pyrazole sulfonamide, possesses fungicidal activity by repressing NMT activity (Fang *et al.* 2015). The virus that was used in this study was a virus that contributed to the 2009 respiratory pandemic, influenza A virus H1N1, has its neuraminidase targeted (PDB: 4B7R, chain A) along with its inhibitor drug attached with the PDB, oseltamivir (van der Vries *et al.* 2012).

Lastly, unrelated to human disease, a study showed that the fungus *Colletotrichum* is harbored in *Rafflesia* (Refaei *et al.* 2011). We targeted the chitin deacetylase enzyme of *Colletotrichum* that contributes to the formation of the fungal cell wall (PDB: 2IW0) with its natural ligand chitin and chitosan (Blair *et al.* 2006), and the 3DLigandSite was used to determine the active sites. This interaction was used to check the possible fungal inhibitory response by *Rafflesia* innate secondary metabolites during browning in plant tissue culture since no fungal growth was observed for several days during *in vitro* culture (Wicaksono and Teixeira da Silva 2015).

Drug-likeness and Bioactive Compound Screening

The SMILES codes of the 21 metabolites were submitted for input in an online drug-likeness test by the Swiss Institute of Bioinformatics: absorption, distribution, metabolism, and excretion test or SwissADME (http://www.swissadme.ch/index.php). This test was carried out to observe initial data, which consisted of molecular weight, chemical pharmacokinetic properties, and drug-likeness properties based on Lipinski *et al.* (1997) and the "rule of five" (LRO5) – including molecular weight ≤ 500 g/mol, octanol-water partition coefficient MLog P ≤ 4.5 (Moriguchi *et al.* 1992), hydrogen bond acceptor (N or O) ≤ 10, and hydrogen bond donor (NH or OH) ≤ 5). The ADME result is listed in the table, together with any violations that occurred. Along with the ADME LRO5, bioavailability defines how the compound can enter the systemic blood circulation and, thus, become available at the site of action (Shargel and Yu 2015) and synthetic accessibility describes the difficulty of the compound to be synthetically manufactured based on its structural complexity (Ertl and Schuffenhauer 2009). Additionally, as supplemental data and as a comparison when LRO5 drug-likeness was not satisfied, other criteria to determine the drug-likeness were used – including those by Ghose *et al.* (1999), Veber *et al.* (2002), Egan *et al.* (2000), and Muegge (2006). Bioavailability and synthetic accessibility data were also included.

Next, the 21 SMILES codes were submitted to the Way2Drug PASS Online website (http://www.way2drug.com/PASSOnline) for screening bioactivity (Lagunin *et al.* 2000). The selected criteria included anticholesterol (cholesterol antagonist, anti-hypercholesterolemic, cholesterol synthesis inhibitor and cholesterol absorption inhibitor), anticancer (anticarcinogenic, antineoplastic, antioxidant, antineoplastic for breast cancer and liver cancer, and angiogenesis inhibitor), anti-neurodegenerative diseases (neurodegenerative disease treatment and acute neurologic disorders treatment), antiviral (general antiviral, general anti-influenza, anti-influenza A, antifungal (general antifungal and chitin synthase inhibitor), wound healing (wound healing and angiogenesis stimulant), and aphrodisiac (estrogen receptor agonists) activities. The resulting data were then tabulated and processed as bar graphs for comparison. These data were used for comparison with ligand-protein affinity results. If the activity likelihood potential (Pa) score was < 0.5, this indicates that the substance is less likely to exhibit activity in an experiment; if 0.5–0.7, the substance is likely to exhibit activity in an experiment; and if > 0.7, it is very likely to exhibit activity in an experiment (Lagunin *et al.* 2000).

Molecular Docking, Visualization, and Molecular Dynamics

Molecular docking was conducted with PyRx–Virtual Screening Tool (Python Prescription 0.8) software (Trott and Olson 2010). The metabolites were first loaded in the Open Babel (O'Boyle *et al.* 2011) tab and had all energy minimized using the universal force field setting, with the optimization algorithm set to "conjugate gradients" and with default settings (200 total steps; number of step

updates = 1; stop if the energy difference was < 0.1). The metabolite ligands with a minimized energy setting were converted to AutoDock Ligand (PDBQT) format for ligand testing. After the tested protein PDB files were loaded, under the Vina Wizard tab, the metabolite ligands were subjected to multiple ligands docking settings (all 21 metabolites simultaneously *vs.* one protein). The Vina search area of each protein was restricted to the area of ligand binding sites of each protein from the RCSB PDB attached ligands or data from 3DLigandSite. Each protein has a different Vina grid box search space and dimension size (Table 1). After the search area for a single protein was determined, docking simulation was initiated. Binding affinity output data was saved as a CSV file. The same protocol was repeated for the other proteins.

For visual observation, all docking output results of the metabolite ligands of each protein (indicated in the AutoDock tab with the "out.pdbqt" format) were saved as a PDB file for visualization and its complex form (ligand-protein complex) was saved into a new PDB file in PyMol Molecular Graphic System v.251 software. The ligand-protein complex file was then loaded to visualize the hydrogen bonds and hydrophobic interactions between the protein amino acid residues and the metabolite ligand molecule using LigPlus (run under Java Runtime Environment) (Laskowski and Swindells 2011) v2.2.4. The process was repeated for the remaining ligand-protein complex files.

Following the docking results, the protein docking complexes with Rafflesiaceae metabolites with the best binding affinity scores were selected and compared with the control ligands. The molecular dynamics between each ligand compound and protein were also simulated using the GROMACS molecular dynamics program (Bekker *et al.* 1993; Abraham *et al.* 2015) and submitted *via* WebGro for the Macromolecular Simulation web server by the University of Arkansas for Medical Sciences (UAMS) SimLab (https://simlab.uams.edu/). Each protein-ligand complex PDB file was submitted to the web server along with the ligand topology file (in ZIP format), generated *via* the GlycoBioChem PRODRG2 Server of the University of Dundee, UK (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg/) (Schüttelkopf and van Aalten 2004). The protein-ligand complex was simulated under the standard (default) setting of GROMOS96 43a1 forcefield with a cubic box type, SPC water model and 0.15 M NaCl salt was added to the system to neutralize the charge. Energy minimization used the steepest descent integrator with 5000 steps. The simulation was run using the NVT/NPT equilibration type, at 300 K, 1 bar, leap-frog integrator, and 1000 frames per simulation for 20 ns. The selected output was protein root-mean-squared deviation (RMSD) per time unit, protein-ligand RMSD per time unit, protein root-mean-squared fluctuation (RMSF) per residue, and protein-ligand hydrogen bond changes per time unit.

Alternative Metabolite Source Review Search

As an additional data set for comparison, the availability of 21 metabolites of *Rafflesia* and *Sapria* was compared to three popular and easily accessed edible plant species for each metabolite. The plant species that were selected are those that are not indexed in any CITES Appendix (listed under https://www.speciesplus.net/species#/) or IUCN Red List (https://www.iucnredlist.org). These plants with the 21 metabolites serve as potential alternatives to *Rafflesia* and *Sapria*, and to determine if the 21 metabolites are available in other plants or Rafflesiaceae-specific metabolites.

RESULTS

Drug-likeness Properties

According to SwissADME screening based on Lipinski's (Lipinski *et al.* 1997) LRO5 drug-likeness (Table 2), all 21 metabolites – including alkaloids, phenolics (six compounds, including aromatic acids), phenylpropanoid

Table 1. PyRx Vina Wizard gridbox search spaces and dimensions.

Protein	Vina search space (binding location)			Dimension		
	X	Y	Z	X	Y	Z
HMG-CoA reductase	41.702	8.463	4.486	28.12	22.845	38.259
Chitin deacetylase from *Colletotrichum*	20.856	5.396	2.974	20.307	20.393	20.873
VEGFR2	–26.415	–0.476	–8.075	23.798	20.731	26.242
Aspergillus fumigatus NMT	–13.999	10.678	–21.068	34.172	29.881	41.797
Alzheimer AcChE	89.048	86.987	–5.26	20.028	23.405	14.287
Influenza A N1 neuraminidase	–26.893	–17.415	–41.185	22.143	25	25.017
Estrogen receptor α	26.268	19.899	20.372	23.026	24.122	33.019
Glycogen synthase kinase 3-b	22.749	26.147	5.603	25	22.761	24.146

Table 2. SwissADME output of the *Rafflesia-Sapria* metabolites.

Metabolite group	Metabolite	Molecular weight (g/mol)	Lipinski's Rule of Five	Bioavailability score	Synthetic accessibility
Alkaloid	Caffeine	194.19	Yes	0.55	2.03
	Nicotine	162.23	Yes	0.55	2.05
Phenolic	Catechin	290.27	Yes	0.55	3.5
	Hydroxybenzoic/ phenolic/ salicylic acid	138.12	Yes	0.85	1
	Leucoanthocyanin	242.27	Yes	0.55	3.44
Hydrolyzable tannin (gallotannin)	1,2,4,6-tetra-*O*-galloyl-β-D-glucose	788.57	No (3 violations[a,c,d])	0.17	5.92
	1,2,6-tri-*O*-galloyl-β-D-glucose	636.47	No (3 violations[a,c,d])	0.17	5.34
	1,4,6-tri-*O*-galloyl-β-D-glucose	636.47	No (3 violations[a,c,d])	0.17	5.32
	1,2,4-tri-*O*-galloyl-β-D-glucose	636.47	No (3 violations[a,c,d])	0.17	5.32
	1,6-di-*O*-galloyl-β-D-glucose	484.36	No (2 violations[c,d])	0.17	4.75
Phenilypropanoid glycoside	Syringin	372.37	Yes	0.55	4.74
Phenolic (anthocyanin pigment)	Cyanidin 3-*O*-glucoside	449.38	No (2 violations[c,d])	0.17	5.3
	Cyanidin 3-*O*-xyloside	419.36	Yes (1 violation[d])	0.55	4.97
	Peonidin 3-*O*-glucoside	463.41	No (2 violations[c,d])	0.17	5.38
Phenolic (flavonol)	Quercetin 3-*O*-glucoside	463.37	No (2 violations[c,d])	0.11	5.28
	Quercetin 7-*O*-glucoside	464.38	No (2 violations[c,d])	0.17	5.31
	Quercetin 3-*O*-glucuronide	478.36	No (2 violations[c,d])	0.11	5.18
	Isorhamnetin 3-*O*-glucoside	478.4	No (2 violations[c,d])	0.17	5.44
Phenolic (aromatic acid)	Ellagic acid	302.19	Yes	0.55	3.17
	Gallic acid	170.12	Yes	0.56	1.22
	Ethyl gallate	198.17	Yes	0.55	1.69

Note: obedience to maximum 1 violation of Lipinski's Rule of Five (RO5): [a]molecular weight ≤ 500 g/mol; [b]Moriguchi octanol-water partition coefficient (M log P) ≤ 4.15; [c]hydrogen bond acceptors (N or O) ≤ 10; [d]hydrogen bond donors (NH or OH) ≤ 5

glycoside, and syringin – obey LRO5. A single anthocyanin phenolic compound, cyanidin 3-*O*-xyloside, also followed LRO5 despite a single violation since the number of hydrogen bond donors exceeded five. Other compound groups in anthocyanin phenolic compounds, flavonols and gallotannins, did not follow LRO5 since the number of hydrogen bond donors and acceptors exceed the limit. For gallotannins, the disagreement with LRO5 was also caused by the large molecular weights except for 1,6-digaloyl-glucose. For comparison, other criteria (Veber, Egan, Ghose, and Muegge) were used (Appendix I Table 2). All anthocyanin phenolics and two flavonols followed the Ghose criteria. A single flavonol, quercetin 3-*O*-xyloside, disobeyed Ghose criteria and – together with the gallotannins – disagree with LRO5 and the remaining criteria – namely, Veber, Egan, and Muegge criteria. The highest bioavailability was shown by salicylic acid (0.85), whereas the lowest bioavailability (0.11) was shown by two flavonols (quercetin 3-glucoside and quercetin 3-glucuronide) (Table 2). Salicylic acid, a phenolic compound, had the lowest synthetic accessibility (1.00) among all 21 compounds, whereas the gallotannins, flavonol phenolics, anthocyanins, and syringin (phenylpropanoid glycoside) had a value between 4–5 (Table 2).

Metabolite Screening for Potentials of Activity

Only 17 of 21 metabolites showed various ranges of scores in bioactivity when tested using PASS Online (Figure 2). Four metabolites (cyanidin 3-glucoside, cyanidin 3-xyloside, peonidin 3-glucoside, and quercetin 3-glucoside) could not be tested by the PASS Online server because they were detected as charged molecules.

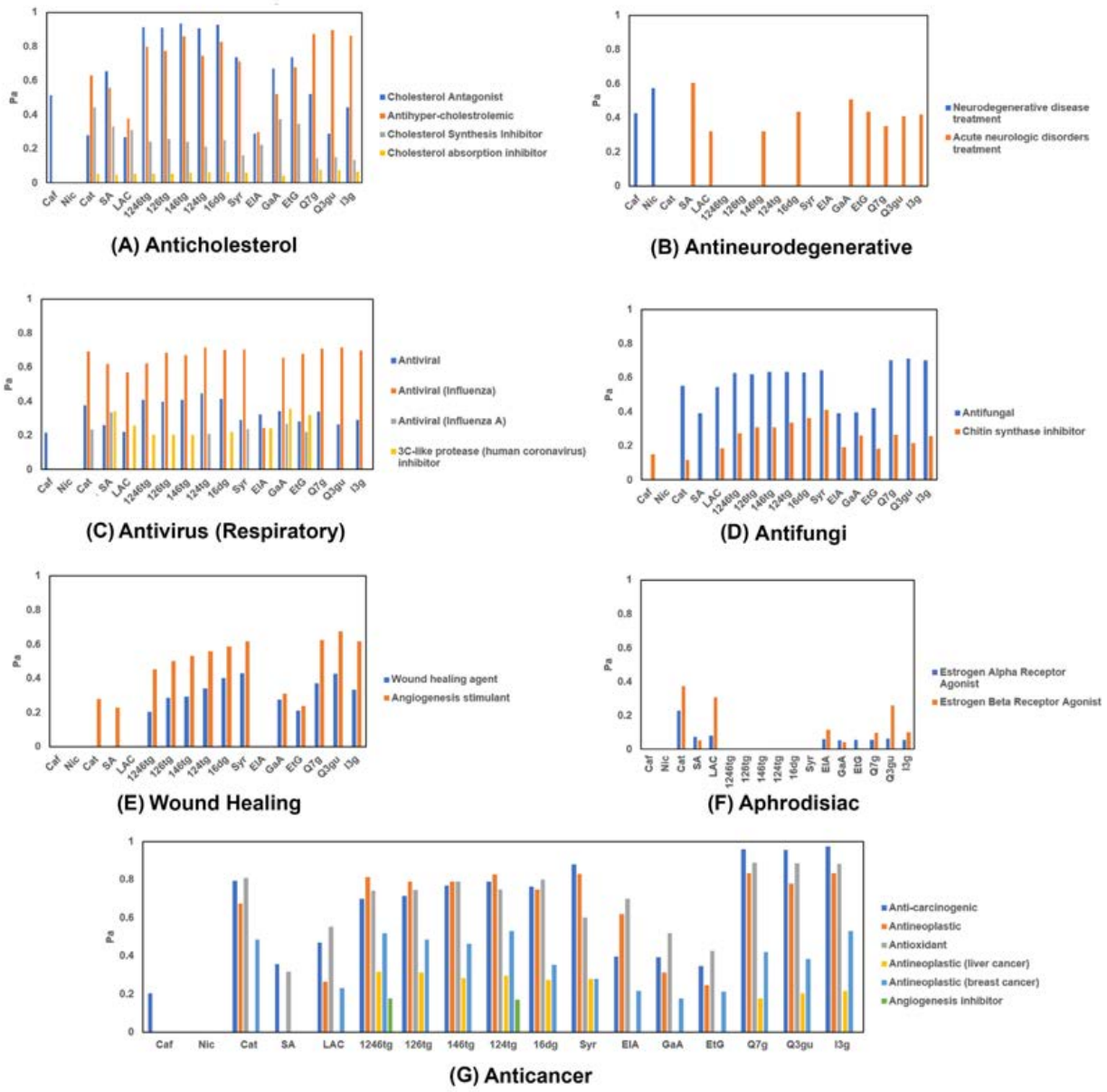

Figure 2. PASS Online drug activity test results on all 17 metabolites in nine categories of bioactivities. Note that the four remaining metabolites could not be tested as they are charged molecules. Abbreviated molecule groups and names: alkaloids (Caf – caffeine; Nic – nicotine), phenolics (Cat – catechin; SA – salicylic acid; LAC – leucoanthocyanidin), gallotannins (1246tg – 1,2,4,6-tetragalloyl glucose; 126tg – 1,2,6-trigalloyl glucose; 146tg – 1,4,6-trigalloyl glucose; 124tg – 1,2,4-trigalloyl glucose; 16dg – 1,6-digalloyl glucose), phenylpropanoid glycoside (Syr – syringin), anthocyanin phenolics (C3g – cyanidin-3-glycoside; C3x – cyanidin-3-xyloside; P3g – peonidin-3-glycoside), flavonol phenolics (Q3gc – quercetin-3-glycoside; Q7g – quercetin-7-glycoside; Q3gu – quercetin-3-glucuronide; I3g – isorhamnetin-3-glycoside), and aromatic acid phenolics (ElA – ellagic acid; GaA – gallic acid; EtG – ethyl gallate).

The PASS Online screening results show that five gallotannins (abbreviations: 1246tg, 124tg, 126tg, 146tg, and 16dg) and three flavonol phenolics (abbreviation: Q7g, Q3gu, and I3g) dominated the high-likeliness scale and were shown to be active (Pa > 0.5) in anticholesterol (mainly as cholesterol antagonists and an anti-hypercholesterolemic), respiratory antiviral (specifically, against influenza virus), antifungi (directly antifungal), wound healing (specifically, as an angiogenesis stimulant), and anticancer (mainly as an anticarcinogenic, antineoplastic, and antioxidant). A phenolic compound, catechin, was also highly anticancer (also anticarcinogenic, antineoplastic, and antioxidant). Only a few are likely to be active as antineurodegenerative

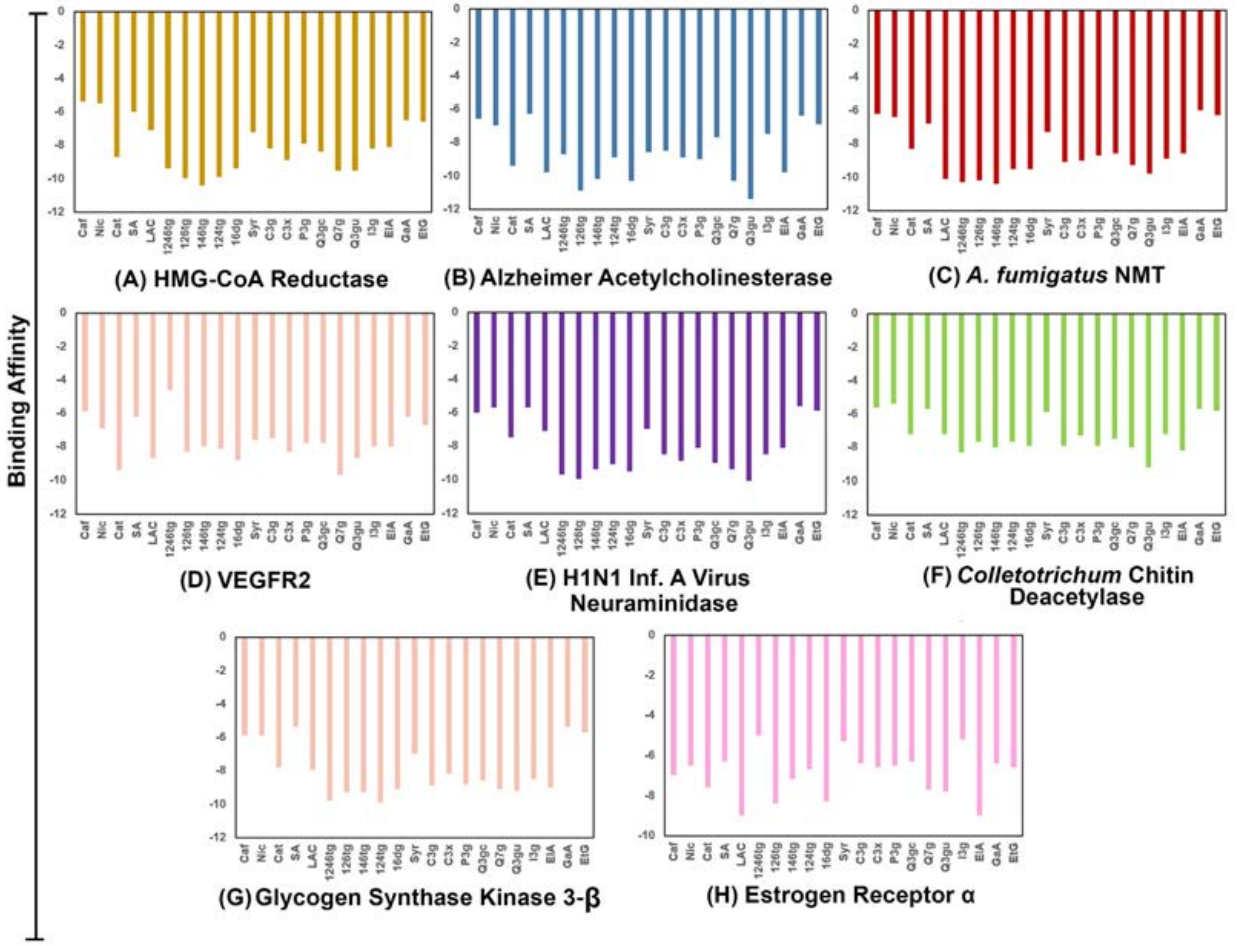

Figure 3. Overview of binding affinities of all 21 metabolites against 11 proteins. The X-axis indicates the molecule names and Y-axis indicates the binding affinities (in kcal/mol). Abbreviated molecule groups and names: alkaloids (Caf – caffeine; Nic – nicotine), phenolics (Cat – catechin; SA – salicylic acid; LAC – leucoanthocyanidin), gallotannins (1246tg – 1,2,4,6-tetragalloyl glucose; 126tg – 1,2,6-trigalloyl glucose; 146tg – 1,4,6-trigalloyl glucose; 124tg – 1,2,4-trigalloyl glucose; 16dg – 1,6-digalloyl glucose), phenylpropanoid glycoside (Syr – syringin), anthocyanin phenolics (C3g – cyanidin-3-glycoside; C3x – cyanidin-3-xyloside; P3g – peonidin-3-glycoside), flavonol phenolics (Q3gc – quercetin-3-glycoside; Q7g – quercetin-7-glycoside; Q3gu – quercetin-3-glucuronide; I3g – isorhamnetin-3-glycoside), and aromatic acid phenolics (ElA – ellagic acid; GaA – gallic acid; EtG – ethyl gallate). See Appendix I Table 3 for a comparison to the control.

(alkaloid nicotine as a neurodegenerative treatment and phenolic salicylic acid). The metabolites displayed low likeliness as an estrogen-binding, aphrodisiac-mimicking component. These activity potentials were then tested under molecular docking simulation.

Molecular Docking

Each protein docked with metabolite ligands in this study was compared to their natural ligands or their standard inhibitory medicines (Table 3), which in docking studies are equivalent to the control or dummy. The binding complex between ligands and proteins was influenced by hydrogen bonds and hydrophobic interactions of the residual amino acids inside the protein (Table 4; see Appendix I Table 3 for details). The docking results (Table 3; Figure 3 for visual comparison) show that in all protein-metabolite ligand complexes, from among nine proteins, six that had the tested metabolites had higher affinities than the control ligands, whereas three proteins have affinities that are all lower than the control ligands.

Of all 21 metabolites (Table 3), the gallotannins (1246tg, 124tg, 126tg, 146tg, and 16dg) had strong binding scores for the anticholesterol target HMGCR, respiratory antiviral targets H1N1 neuraminidase, and wound-healing GSK-3β. The same scores were also held by the phenolic, catechin. Phenolic anthocyanins (C3g, C3x, and P3g) and flavonols (Q3g, Q3gu, Q7g, and I3g) also had high binding scores to respiratory antiviral targets H1N1 neuraminidase and wound-healing GSK-3β. Exceptionally, one flavonol quercetin-3-glucuronide (Q3gu) tops the binding scores

Table 3. Binding affinities of control compounds (natural ligands or patented drugs) to 11 proteins on docking using PyRx – Autodesk Vina, selected with upper and lower root-mean-square deviation (RMSD) of each result equal 0.

Protein	Compounds	Molecular weight (g/mol)	Binding affinity (kcal/mol)
3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA) reductase	Atorvastatin	558.64	–8.5
	3-hydroxymethylglutaric Acid (HMG)	160.12	–5.6
	Coenzyme A (CoA)	755.44	–8.2
Chitin deacetylase from *Colletotrichum*	Chitin	608.44	–9.3
Vascular endothelial growth factor receptor 2 (VEGFR2)	Pyrrolopyrimidine	445.37	–12.4
	Sorafenib	464.82	–12.3
Aspergillus fumigatus N-myristoyltransferase (NMT)	Myristoyl-CoA	977.89	–8
	Pyrazole sulfonamide	537.51	–10.6
Alzheimer acetylcholinesterase	Donepezil	379.49	–11.3
Influenza A H1N1 neuraminidase	Oseltamivir	234.61	–6.9
Estrogen receptor α	WAY-244	265.26	–9.5
Glycogen synthase kinase 3	TMU	303.31	–7.6

in anticholesterol target HMGCR, antineurodegenerative target against Alzheimer AchE, respiratory antiviral targets H1N1 neuraminidase, and wound-healing GSK-3β – making it highly prospective as a potential inhibitory drug. No metabolites were able to bind more strongly than control ligands among the antifungal targets (*A. fumigatus* NMT and *Colletotrichum* chitin deacetylase), anticancer target (anti-breast cancer, VEGFR2 protein), and aphrodisiac estrogen-mimicking target of estrogen receptor α.

In terms of protein-ligand interactions (Appendix I Table 3; Figure 4 for protein–inhibitory drug/natural ligand interactions, the remaining protein-ligand interactions viewed using LigPlus in Appendix II), there are hydrogen bonds and hydrophobic interactions that are specific to each complex and these interactions contribute to the high binding affinity scores.

Molecular Dynamics

The results of molecular dynamics simulations using GROMACS revealed the dynamics of interactions between the complexes of proteins and ligands, both against control ligands and against tested Rafflesiaceae metabolites (Appendix I Figure 2). The metabolite that docked to HMGCR (Appendix I Figure 2A) with the best docking binding affinity is 1,4,6-trigalloyl glucose. Both atorvastatin (total average RMSD throughout the simulation period: 0.3 nm) and 1,4,6-trigalloyl glucose had higher RMSD than the control ligand, especially after 5 ns of simulation, at which point the RMSD increased further (before 5 ns: 0.4–0.6 nm, after: 0.6–1.0 nm in 1,4,6-trigalloyl glucose). The HMGCR-atorvastatin complex had a stable RMSD fluctuation after 5 ns at 0.2–0.4 nm. Without the ligands, HMGCR protein dynamics appeared to be stable around 0.3–0.35 nm of RMSD after 5 ns (observed in two repetitions during the two-ligands simulation), implying that upon docking, the change in RMSD was still in the RMSD range of the protein without any ligand. The RMSF of both the metabolite ligand and control appears to be consistent with both chains A and B (< 0.3 nm, except for some high fluctuations at the protein end residues) and the hydrogen bonds fluctuated between one and two hydrogen bonds in both ligands. For these results, 1,4,6-trigalloyl glucose may still be a good and stable HMGCR inhibitor compared to atorvastatin, despite the differences in RMSD, because the fluctuation is somewhat stable. Following docking with all metabolites, the GSK-3β proteins (Appendix I Figure 2B) showed 1,2,4-trigalloyl glucose as having the best binding affinity. In addition, it also has a more stable RMSD – even before 5 ns – than its control ligand (TMU), which showed high fluctuations, even until 15 ns. Without the ligands, despite some slight differences in fluctuations, RMSD was stable between 0.2–0.3 ns. The protein-TMU complex RMSD was initially close to the protein-only RMSD (0.4–0.8 nm before 10 ns of simulation), but it deviated further to around 1.2–1.4 nm after 10 ns. The protein-tested metabolite complex was more stable, with RMSD almost flat to 0.8 nm. The RMSF values of the protein upon docking with either the control or the metabolite ligand were consistent and less than 0.4 nm. Compared to the control, 1,2,4-trigalloyl glucose had more hydrogen bonds during simulation (three bonds on average, whereas TMU has only one bond on average).

The proteins of estrogen receptor α (Appendix I Figure 2C) indicated ellagic acid as the metabolite with the best affinity. After 5 ns, ellagic acid had a lower RMSD

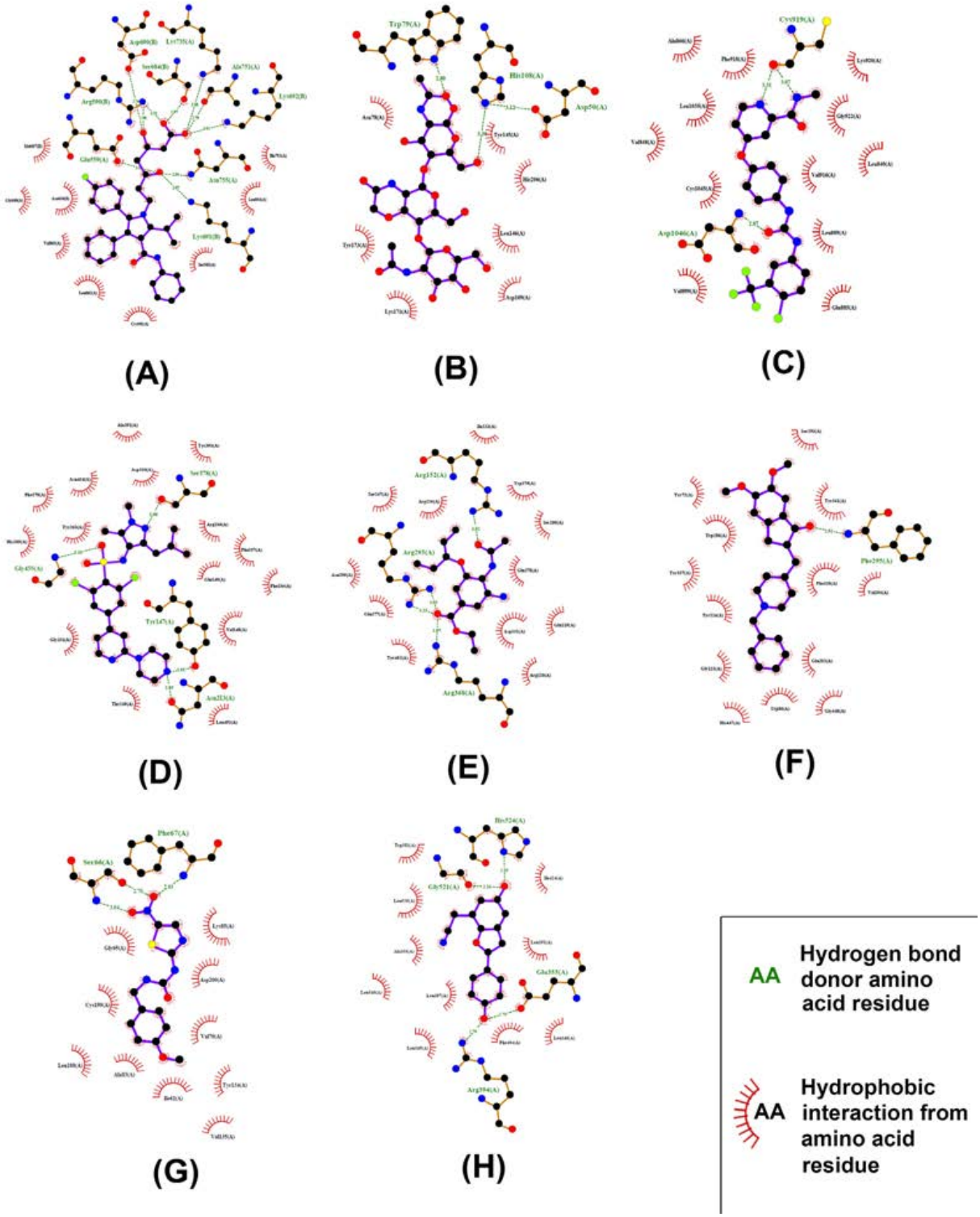

Figure 4. Proteins–inhibitory drug/natural ligand complexes: HMGCR–atorvastatin (A), *Colletotrichum* chitin deacetylase–chitin (B), VEGFR2–sorafenib (C), NMT–pyrazole sulfonamide (D), H1N1 neuraminidase single unit–oseltamivir (E), acetylcholinesterase–dopafenib (F), glycogen synthase kinase 3-β–TMU (G), and estrogen receptor α–WAY244 (H). The order of proteins in this figure follows Figure 1. Viewed in LigPlus v2.2.4. The remaining protein-metabolite ligand interactions are listed in Appendix II.

Table 4. Binding affinities of 21 *Rafflesia-Sapria* metabolites to 11 proteins on docking using PyRx – Autodesk Vina, selected with upper and lower root-mean-square deviation (RMSD) of each result equal 0.

Metabolite group	Metabolite	Molecular weight (g/mol)	Binding affinity (kcal/mol; at upper and lower bound RMSD = 0)							
			HMG-CoA reductase (Site 1/ Chain A and B)	Alzheimer acetylcholinesterase (Chain A)	*Aspergillus fumigatus* NMT protein	VEGFR2	Influenza A H1N1 neuraminidase (Chain A)	*Colletotrichum* chitin deacetylase	Estrogen receptor α (Chain A)	Glycogen synthase kinase 3-b (Chain A)
Alkaloid	Caffeine	194.19	–5.4	–6.6	–6.2	–5.9	–6	–5.6	–7	–5.9
	Nicotine	162.23	–5.5	–7	–6.4	–6.9	–5.7	–5.4	–6.5	–5.9
Phenolic	Catechin	290.27	–8.7	–9.4	–8.3	–9.4	–7.5	–7.2	–7.6	–7.8
	Hydroxybenzoic/ phenolic/ salicylic acid	138.12	–6	–6.3	–6.8	–6.2	–5.7	–5.7	–6.3	–5.4
	Leucoanthocyanin	242.27	–7.1	–9.8	–10.1	–8.7	–7.1	–7.2	–9	–7.9
Hydrolyzable tannin (gallotannin)	1,2,4,6-tetra-*O*-galloyl-β-D-glucose	788.57	–9.4	–8.7	–10.3	–4.6	–9.7	–8.3	–5	–9.8
	1,2,6-tri-*O*-galloyl-β-D-glucose	636.47	–10	–10.9	–10.2	–8.3	–10	–7.7	–8.4	–9.3
	1,4,6-tri-*O*-galloyl-β-D-glucose	636.47	–10.4	–10.2	–10.4	–8	–9.4	–8	–7.2	–9.3
	1,2,4-tri-*O*-galloyl-β-D-glucose	636.47	–9.9	–8.9	–9.5	–8.1	–9.1	–7.7	–6.7	–9.9
	1,6-di-*O*-galloyl-β-D-glucose	484.36	–9.4	–10.3	–9.5	–8.8	–9.5	–7.9	–8.3	–9.1
Phenilypropanoid glycoside	Syringin	372.37	–7.2	–8.6	–7.3	–7.6	–7	–5.9	–5.3	–7
Phenolic (anthocyanin pigment)	Cyanidin 3-*O*-glucoside	449.38	–8.2	–8.5	–9.1	–7.5	–8.5	–7.9	–6.4	–8.9
	Cyanidin 3-*O*-xyloside	419.36	–8.9	–8.9	–9	–8.3	–8.9	–7.3	–6.6	–8.2
	Peonidin 3-*O*-glucoside	463.41	–7.9	–9	–8.7	–7.8	–8.1	–7.9	–6.5	–8.8
Phenolic (flavonols)	Quercetin 3-*O*-glucoside	463.37	–8.4	–7.7	–8.6	–7.8	–9	–7.5	–6.3	–8.6
	Quercetin 7-*O*-glucoside	464.38	–9.5	–10.3	–9.3	–9.7	–9.4	–8	–7.7	–9.1
	Quercetin 3-*O*-glucuronide	478.36	–9.5	–11.4	–9.8	–8.7	–10.1	–9.2	–7.8	–9.2
	Isorhamnetin 3-*O*-glucoside	478.4	–8.2	–7.5	–8.9	–8	–8.5	–7.2	–5.2	–8.5
Phenolic (aromatic acid)	Ellagic acid	302.19	–8.1	–9.8	–8.6	–8	–8.1	–8.2	–9	–9
	Gallic acid	170.12	–6.5	–6.4	–6	–6.2	–5.6	–5.7	–6.4	–5.4
	Ethyl gallate	198.17	–6.6	–6.9	–6.3	–6.7	–5.9	–5.8	–6.6	–5.7

(0.2 nm on average) upon interaction with the protein compared to the control ligand (WAY-244) (0.4 nm on average), indicating higher stability. The protein RMSDs without ligands were consistent after 5 ns and between 0.2–0.3 nm, so the docked protein with ellagic acid had the closest RMSD value (*i.e.* it did not change much) to the protein RMSD (without ligand). Upon contact, there were two residue regions (between residues 305–355 and 455–505) with a highly fluctuating RMSF, but the remaining residues were on average stable between 0.05–0.2 nm. Similarly, both ellagic acid and control ligands had almost stable hydrogen bonds throughout the simulation at one bond. The *Colletotrichum* fungal chitin deacetylase (Appendix I Figure 2D) bonded with quercetin 3-glucuronide with a lower fluctuation in RMSD (< 1 nm) than the natural ligand itself, *i.e.* the control [chitin (between 1 and 2 nm)], making quercetin 3-glucuronide a good inhibitor candidate. The blank protein RMSD, however, fluctuated between 0.15–0.4 nm. Thus, the quercetin bonding RMSD value was closer to the blank protein (less fluctuation than the chitin RMSD value). On the other hand, the RMSF of the protein was < 0.3 nm with no highly fluctuating residue. Despite the low RMSD profile, quercetin 3-glucuronide had fewer hydrogen bonds per total unit time (around one bond) compared to the control (2–3 bonds). Although the difference in the number of hydrogen bonds, in this case, was considerable – because chitin is a large macromolecule that tends to have more interactions with protein residues than if the ligand molecule were small – only the oligomer was used in this simulation.

For VEGFR2 (Appendix I Figure 2E), the protein-ligand RMSD for the best metabolite among all 21 tested metabolites, *i.e.* quercetin 7-glucoside, was 0.15–0.3 nm, whereas that of the control ligand (pyrrolopyrimidine) was 0.25–0.4 nm. Comparatively, the RMSD of the protein itself ranged between 0.25–0.35 nm after 5 ns for both simulations. Hence, the changes in RMSD of the docked protein were generally still close to the range of the RMSD of only the protein. Between residues 911–1011, the fluctuations were higher (up to 0.5 nm), whereas the remainder were less than 0.3 nm. The hydrogen bonds in contact with quercetin 7-glucoside were higher (two bonds were formed most of the time) than the control (dominantly one bond). This indicates that quercetin 7-glucoside could bind better than pyrrolopyrimidine to the protein. For the neuraminidase of influenza H1N1 virus (Appendix I Figure 2F), the RMSD of the control ligand (oseltamivir) was lower (< 0.8 nm) than the competing ligand (quercetin 3-glucuronide) (0.8–1.6 nm), making oseltamivir close to the RMSD range (≤ 0.3 nm) of the protein without ligand. The protein RMSF values (during both simulations with oseltamivir and quercetin 3-glucuronide) were not larger than 0.3 nm, implying that the protein interactions were relatively stable, with only a few fluctuating peaks. There was one exception, a peak that almost reached 0.5 nm, located between residues 83–183. The hydrogen bonds for both oseltamivir and quercetin 3-glucuronide were relatively similar, dominated by only one bond. However, during 20 ns of simulation, the protein residues around quercetin 3-glucuronide had more interactions, temporarily increasing the number of hydrogen bonds at some moments to 2.

For the *Aspergillus flavus* NMT protein (Appendix I Figure 2G), the best docking result was with 1,4,6-trigalloyl glucose. However, the docking RMSD profile was close to that of the control (on average, 0.4–0.6 nm; in comparison, the RMSD range of the blank protein was 0.2–0.35 nm). Even though the RMSF value was generally less than 0.4, there were some fluctuating peaks that reached 0.4 nm. For N-myristoyl-CoA, the control, hydrogen bonds were dominantly 3 and for 1,4,6-trigalloyl glucose, hydrogen bonds were dominantly 2 or 3, although in both, the maximum number of bonds that formed was seven. Finally, for the protein commonly used as a drug target for Alzheimer's disease, acetylcholine esterase (Appendix I Figure 2H), quercetin 3-glucuronide was the best docking metabolite among all 21 docked metabolites. However, the RMSD value of quercetin 3-glucuronide was much higher (≤ 14 nm) than the control (donepezil, with an RMSD value of < 0.5 nm) and the protein by itself (range: 0.2–0.3 nm). Although the average RMSF value was generally less than 0.3 nm (except for a few fluctuating residues), the number of hydrogen bonds for both the control and competing ligand was low (dominantly 1, but occasionally 2) and formed late (after 5 ns). In this case, quercetin 3-glucuronide is not a good candidate because of its especially high RMSD and late (> 10 ns) hydrogen bond formation time.

Alternative Sources

Based on a literature search, there appear to be many alternative sources for all metabolites (Appendix I Table 4). For example, tea (*Camelia sinensis* L.) has caffeine (alkaloid), catechin, and salicylic acid (phenolics), whereas apple (*Malus domestica* Borkh) has leucoanthocyanidin (phenolics) and quercetin 3-glucoside (phenolics: flavonol). The anthocyanin phenolic pigments appear to be consistent in red and purple-colored fruits, such as strawberry (*Fragaria* × *ananassa* Duchesne), cranberry (*Vaccinium macrocarpon* Aiton), blackberry (*Rubus fruticosus* L.), and blueberry (*Vaccinium* sp.). Among all, however, the Rafflesiaceae-characterized gallotannins appear to be available only in specific and less common plants. No literature indicated any fruits, vegetables, or local medicinal plants with 1,2,4-trigalloyl glucose. Therefore, all common and edible plants can serve as alternative sources for the 21 metabolites that

were found in the two Rafflesiaceae genera of this study. Only gallotannins require additional attention for metabolite prospecting if all metabolites are planned to be artificially mixed in a Rafflesiaceae plant-based medicine.

DISCUSSION

Drug Potential

Based on ethnomedicinal claims, some species in the Rafflesiaceae are used by locals, primarily in Indonesia and Malaysia, to cure some diseases. In prospective medicinal studies, *Rafflesia* extracts have been claimed to possess antioxidant (Puttipan and Okonogi 2014; Zulkffle *et al.* 2014; Bakoush *et al.* 2015; Chuangchot *et al.* 2017; Norhazlini *et al.* 2021), antifungal (Wiart *et al.* 2004), and wound-healing (Abdulla *et al.* 2009) properties. On the other hand, *Rhizanthes* extracts are rich in antioxidants, serving as a biopesticide, tested by a lethal dosage test on brine shrimp (Saleh *et al.* 2015). However, no study to date has revealed the medicinal properties of *Sapria*, although there are some compounds that are similar to those in *Rafflesia* and that were used in this study (Appendix I Table 1).

No details on *Rhizanthes* metabolites are available in the literature, whereas the metabolites of only *Rafflesia* and *Sapria* have been partially or superficially characterized. *In silico* screening with PASS Online (Figure 2) confirmed the antioxidant property of *Rafflesia* and *Sapria* metabolites. The gallotannins and flavonol phenolics possessed a high likelihood of anticholesterol, antifungal, and anti-influenza virus properties. For screening anticancer activity, other than being strong antioxidants, the gallotannins and flavonol phenolics are highly antineoplastic and anticarcinogenic. These activities were displayed by a single flavonol phenolic (quercetin-7-glucoside) and by a single phenolic (catechin). For the antineurodegenerative property, salicylic acid (a phenolic) is likely ($0.5 > Pa > 0.7$) – to be active in acute neurological disorder treatment and nicotine (an alkaloid) – is likely to be active for neurodegenerative disease treatment. The analysis by PASS Online (Filimonov *et al.* 2014) applies a principle based on structural analysis for structure-activity relationships in a machine learning-based training set, with more than 300,000 organic compounds as the input, of 50–1250 Da in size. The data set contains information on structure *versus* biological activity – including drug substances – drug candidates in various stages of pre-clinical and clinical investigation, pharmaceutical agents, chemical probes, and compounds with known toxicity data to extrapolate the compound's likely activity.

Despite the good probability of activity in PASS Online of the gallotannins and flavonol phenolics for anticholesterol, antifungal, and anti-influenza virus drug prospecting, these compounds did not obey the LRO5 for drug-likeness (Table 1). On the other hand, the flavonol phenolics (except for quercetin 3-glucuronide) passed the Ghose filters (Appendix I Table 2). Based on the drug-likeness screening bioavailability score, the gallotannins possessed low scores (< 0.2 or 20%), as did flavonol phenolics and anthocyanins (except for cyanidin 3-xyloside, which had a score of 0.55 or 55%). Bioavailability implies the speed by which a metabolite reaches the systemic bloodstream (Shargel and Yu 2015) using a method other than intravenous administration, such as oral administration, so a drug delivery method is required (Chan and Stewart 1996). Otherwise, under certain doses and considerations, that metabolite can be administered intravenously. Incidentally, for intravenous administration, bioavailability is 1.00 or 100% (Flynn 2008).

Metabolite Prospects Based on Binding Affinities, Pass Screening, and Molecular Dynamics

In other studies on the medical properties of compounds, alkaloids as salts (as products of the reaction between alkaloids and organic acids) have been used for many drugs – including for the treatment of antiarrhythmic-, antiprotozoal-, antitumor-, antitussive-, and aphrodisiac-related conditions (Hesse 2002), or treatment for Alzheimer's disease in the case of nicotine (Sahakian *et al.* 1989). Phenolics – compounds that encompass the anthocyanin pigments, flavonols, and aromatic acids – are known for their hepatoprotectant (Saha *et al.* 2019), antioxidant (Chu *et al.* 2002), and anticancer (Dai and Mumper 2010) properties. Phenylpropanoid glycoside has been used as a hepatoprotectant, anti-inflammatory, and antioxidant (Lanza *et al.* 2001; Díaz *et al.* 2004; Lin *et al.* 2006). Gallotannins have shown anti-inflammatory, antimicrobial, antitumor, and antioxidant properties (Wu-Yuan *et al.* 1988; Feldman *et al.* 2001; Bakondi *et al.* 2004; Zhao *et al.* 2005; Fawole *et al.* 2009; Park *et al.* 2015; Kiss and Piwowarski 2018). Hence, given that the Rafflesiaceae contain these metabolites, this might explain some of the medicinal effects of some compositions. However, these metabolites can be obtained from other plant sources, as listed in Appendix I Table 4.

The docking results, when combined with the PASS Online screening results, indicated that some of the Rafflesiaceae metabolites were prospective candidates as anticholesterol (based on HMGCR docking) or as an antineurodegenerative (based on AchE docking). They are also slightly prospective candidates as an antifungal because they worked against *A. fumigatus* NMT but not against *Colletotrichum* chitin deacetylase. Additionally,

the Rafflesiaceae metabolites functioned as an antiviral against H1N1 influenza A neuraminidase and displayed an angiogenesis stimulant property that helps in wound healing. However, the metabolite docking VEGFR2 scores were still far below those of the patent drugs pyrrolopyrimidine and sorafenib. The only metabolites in terms of docking results that were superior to these drugs are catechin and quercetin-7-glucoside. Similarly, no compounds' binding affinity was higher than the natural ligand in terms of their ability to bind to estrogen receptor α, as confirmed by the PASS results.

When docked with the *Colletotrichum* chitin deacetylase protein, quercetin 3-glucuronide – a flavonol phenolic – had the strongest chitin-binding affinity compared to the control ligands (chitin and chitosan). In the *Rafflesia* tissue culture study by Wicaksono and Teixeira da Silva (2015), it was suggested that browning for several days inhibited microbial growth. It is possible that microbial inhibition occurred due to the leakage of gallotannins and flavonol phenolics into tissue culture media for several days, thereby inhibiting microbial contaminants until the metabolites apparently degraded and the contaminants started to proliferate, although this hypothesis would need stringent assays to test its validity. Medium browning in plant tissue culture is often caused by the exudation of phenolic compounds, especially polyphenol oxidase (Mayer 1986), whereas leucoanthocyanidin is involved in the formation of oxidized tannins (Roux and Evelyn 1958). However, the binding affinity of leucoanthocyanidin in this study was lower than that of chitin deacetylase, despite its promising PASS Online results for antifungal activity. Presumably, leucoanthocyanidin has a weak or no interference with fungal inhibition, suggesting that the antifungal property may be the combined effect of leucoanthocyanidin and other metabolites.

Most of the molecular dynamics simulation results of the 21 best metabolites are good compared to the control ligands. However, among them, quercetin 3-glucuronide performed poorly as a protein-ligand complex in the molecular dynamics simulation of its docked protein, acetylcholine esterase. This was because the RMSD value of quercetin 3-glucuronide rose continuously and did not stabilize at a certain RMSD distance compared to the RMSD value of the protein and control ligand, donepezil, which rose less than 0.1 nm or even compared to the RMSD value of the protein without the ligand (0.15–0.3 nm). Its RMSD value rose from 0 nm to around 4 nm during the first ns of simulation and increased more than 10-fold by the end of the simulation period (20 ns), reaching 14 nm, whereas the control remained less than 0.15 nm all the time. Additionally, the period of hydrogen bond formation was also later than 10 ns. This indicates that despite good performance in a molecular docking simulation, performing a follow-up molecular dynamics simulation is important to verify the interaction between the ligand and the protein.

Quest for a Rafflesiaceae-based Drug: Natural and Synthetic Prospects

Another aspect of drug-likeness that is assessed by SwissADME screening is synthetic accessibility, in which 0 indicates that the drug can be easily synthesized, whereas 10 indicates that it is difficult to synthesize in a metabolic pathway, and most cataloged bioactive molecules tend to be lower than 6 (Ertl and Schuffenhauer 2009). In this study, the gallotannins and flavonol phenolics proved to be the best drug metabolite candidates, given their many associated medicinal properties. However, the problem is that both gallotannins and flavonol phenolics had moderate synthetic accessibility scores (> 5, except for gallotannin 1,6-digalloyl glucose, with a score of 4.75), signifying a moderate level of difficulty in biosynthesis. These scores are likely due to the molecular structural complexity of gallotannins and flavonol phenolics.

The metabolites found in Rafflesiaceae in this study are apparently also widely available in other domesticated plant sources (Appendix I Table 5) – except for gallotannins, which originate from uncommon plants and 1,2,4-trigalloyl glucose – for which no plant metabolite-related literature exists. This implies that most of the currently identified metabolites are likely not unique to Rafflesiaceae, so a future study should endeavor to identify metabolites specific to this family and attempt to synthesize them in order to better appreciate the active factors that may underlie their ethnomedicinal properties.

To that end, this study may be useful to assist with Rafflesiaceae species genomic characterization, although a large limitation is the incomplete characterization of secondary metabolite pathways, such as for salicylic acid and ellagic acid (see Appendix I Figure 1), so this requires future characterization. We point to an inspiring project that is being performed by the GreatBay United Team of iGEM Competition in 2021 (https://2021.igem.org/Team:GreatBay_United) on the rare horseshoe crab (*Limulus polyphemus* (Linnaeus 1758), where synthetic biology is used to artificially produce the bacterial endotoxin detector component, *Limulus* amoebocyte lysate, to prevent a decline in natural crab populations. Similarly, with additional research, following their accurate isolation and identification using techniques such as MS-GC, HPLC, *etc.*, Rafflesiaceae-specific metabolites can be artificially produced.

Finally, we note that the result of metabolite screening in this study is based on molecular docking and PASS Online, which are highly broad methods. Interactions between metabolites can either be synergistic or antagonistic, and even with docking, their affinity can be screened in PASS

Online, but their activity-inactivity can vary widely. To overcome this limitation, *in vitro* and *in vivo* clinical tests are needed to verify the results.

Possible Solutions and Prospects

This study shows that despite several ethnomedicinal claims made in the Rafflesiaceae literature (see Introduction), there are other sources from domesticated edible plants for 19 out of the 21 metabolites that were assessed. Since many Rafflesiaceae are rare or endangered species, their use for compound extraction is not viable or sustainable, especially the 19 metabolites commonly found in other plant species. However, any future discovery research that identifies unique metabolites with unique medicinal properties would benefit from the use of biotechnologies, such as *in vitro* tissue culture, to mass-produce biomass or bioreactors to multiply metabolites. In such cases, genes and proteins also need to be better characterized, but that is only possible once sufficient sterilized *in vitro* material can be produced since such discovery is not viable using sampling of naturally harvested genetic resources. These technologies, including the eventual ability to synthesize Rafflesiaceae-specific metabolites using transgenic or synthetic biology approaches, aim to preserve natural, wild Rafflesiaceae populations. To date, only three Rafflesiaceae genera – mainly *Rafflesia* – have been the subject of developmental studies (Nikolov *et al.* 2013; Wicaksono *et al.* 2017; Mursidawati *et al.* 2019) or propagation/micropropagation research (Wicaksono and Teixeira da Silva 2015; Sukamto 2001; Sukamto and Mujiono 2010; Mursidawati and Handini 2009; Mursidawati *et al.* 2015; Wicaksono *et al.* 2016; Molina *et al.* 2017). Thus far, only host grafting has shown to be a successful method to propagate *Rafflesia* (Mursidawati *et al.* 2015), and only one moderately successful attempt to tissue culture *Rafflesia* exists, but this only resulted in callus formation, but no organogenesis (Sukamto and Mujiono 2010).

CONCLUSION

Several ethnomedicinal properties, such as wound-healing or aphrodisiac, have been claimed in select literature on Rafflesiaceae species, but no clinical research exists to support these claims. This study tested 21 metabolites that have been characterized in the literature – encompassing alkaloids, phenolics, gallotannins, and phenylpropanoid glycoside – identifying eight proteins associated with diseases – including six human pathogenic-associated HMGCR (cholesterol synthesis), VEGFR2 (breast cancer), NMT (*Aspergillus fumigatus*), neuraminidase (2009 pandemic-causing H1N1 influenza A), GSK3-β (wound healing), and estrogen receptor α – as well as one plant-pathogenic associated *Colletotrichum* chitin deacetylase. Gallotannins and flavonol phenolics had the highest inhibitory effects among the proteins, as confirmed by activity screening using PASS Online, molecular docking, and molecular dynamics. Future studies on Rafflesiaceae need to identify genus-specific metabolites, endeavoring to isolate them and mass-produce plant material using advanced biotechnologies in order to extract a sufficient amount of material to artificially synthesize useful metabolites *en masse*. Were this to be achieved, then the safety of natural populations of endangered Rafflesiaceae could be ensured.

ACKNOWLEDGMENTS

We thank Adriane Tobias (University of the Philippines) for providing the law and regulation for biodiversity protection in the Philippines and Syarifah Haniera Syekh Kamal (Universiti Teknologi MARA, Malaysia) for providing the law and regulation for biodiversity protection in Malaysia. We are also thankful to Viol Dhea Kharisma (Division of Molecular Biology and Genetics, Genbinesia Foundation, Indonesia) for some insight on molecular docking and herbal drug design. Some information from this study was presented at the Philippine Society for Developmental Biology 13th Annual Scientific Conference 2021 (17–19 November, online).

STATEMENT ON CONFLICT OF INTEREST

All authors declare no conflicts of interest.

NOTES ON APPENDICES

Some supplementary data generated or analyzed during this study are included in this published article and are stored by the authors and available online (for Appendix II, accessible *via* https://10.5281/zenodo.6796409).

REFERENCES

ABDULLA MA, AHMED KA, ALI HM, NOOR SM, ISMAIL S. 2009. Wound healing activities of *Rafflesia hasseltii* extract in rats. J Clin Biochem Nutr 45(3): 304–308.

ABRAHAM MJ, MURTOLA T, SCHULZ R, PÁLL S, SMITH JC, HESS B, LINDAHL E. 2015. GROMACS: high performance molecular simulations through

multi-level parallelism from laptops to supercomputers. SoftwareX 1: 19–25.

ALAM S, KHAN F. 2018. Virtual screening, docking, ADMET and system pharmacology studies on *Garcinia* caged xanthone derivatives for anticancer activity. Sci Rep 8: 1–16.

BAKONDI E, BAI P, ERDÉLYI K, SZABÓ C, GERGELY P, VIRÁG L. 2004. Cytoprotective effect of gallotannin in oxidatively stressed HaCaT keratinocytes: the role of poly (ADP-ribose) metabolism. Exp Dermatol 13(3): 170–178.

BAKOUSH SMM, YAACOB WA, ADAM JH, IBRAHIM N. 2015. Antioxidant activities, total phenolic and flavonoid contents of the aqueous extracts from *Rafflesia cantleyi* bud parts. Res J Med Plant 9(7): 347–353.

BÄNZIGER H. 1991. Stench and fragrance: unique pollination lure of Thailand's largest flower, *Rafflesia kerrii* Meijer. Nat Hist Bull Siam Soc 39: 19–52.

BEKKER H, BERENDSEN HJC, DIJKSTRA EJ, ACHTEROP S, VONDRUMEN R, VAN DER SPOEL D, SIJBERS A, KEEGSTRA H, RENARDUS MKR. 1993. Gromacs: a parallel computer for molecular-dynamics simulations. In: International Conference on Computational Physics (PC 92). de Groot RA, Nadrchal J ed. Singapore: World Scientific Publishing. p. 252–256.

BHAT R, XUE Y, BERG S, HELLBERG S, ORMÖ M, NILSSON Y, RADESÄTER AC, JERNING E, MARKGREN PO, BORGEGÅRD T, NYLÖF M. 2003. Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. J Biol Chem 278(46): 45937–45945.

BHATNAGAR RS, FÜTTERER K, FARAZI TA, KOROLEV S, MURRAY CL, JACKSON-MACHELSKI E, GOKEL GW, GORDON JI, WAKSMAN G. 1998. Structure of N-myristoyltransferase with bound myristoylCoA and peptide substrate analogs. Nat Struct Biol 5: 1091–1097.

BLAIR DE, HEKMAT O, SCHÜTTELKOPF AW, SHRESTHA B, TOKUYASU K, WITHERS SG, VAN AALTEN DM. 2006. Structure and mechanism of chitin deacetylase from the fungal pathogen *Colletotrichum lindemuthianum*. Biochemistry 45(31): 9416–9426.

BOUTIN JA. 1997. Myristoylation. Cell Signal 9(1): 15–35.

CHAN OH, STEWART BH. 1996. Physicochemical and drug-delivery considerations for oral drug bioavailability. Drug Discov 1(11): 461–473.

CHU YF, SUN JIE, WU X, LIU RH. 2002. Antioxidant and antiproliferative activities of common vegetables. J Agric Food Chem 50(23): 6910–6916.

CHUANGCHOT C, TATTAWASART U, SRIPANIDKULCHAI B, JUNLATAT J, FANGKRATHOK N. 2017. Antibacterial and antioxidant activity of *Rafflesia kerrii* extract against multidrug-resistant bacteria. Songklanakarin J Sci Technol 39(2): 163–170.

DAI J, MUMPER RJ. 2010. Plant phenolics: extraction, analysis, and their antioxidant and anticancer properties. Molecules 15(10): 7313–7352.

DEPARTMENT OF NATIONAL PARKS, WILDLIFE, AND PLANT CONSERVATION. 2017. Threatened Plants in Thailand. Retrieved on 02 Jul 2022 from http://www.dnp.go.th/botany/PDF/publications/ThreatenedPlantsInThailand.pdf

DÍAZ AM, ABAD MJ, FERNÁNDEZ L, SILVÁN AM, DE SANTOS J, BERMEJO P. 2004. Phenylpropanoid glycosides from *Scrophularia scorodonia*: *in vitro* anti-inflammatory activity. Life Sci 74(20): 2515–2526.

EGAN WJ, MERZ KM, BALDWIN JJ. 2000. Prediction of drug absorption using multivariate statistics. J Med Chem 43(21): 3867–3877.

ERTL P, SCHUFFENHAUER A. 2009. Estimation of synthetic accessibility score of drug-like molecules based on molecular complexity and fragment contributions. J Cheminform 1: 8.

FANG W, ROBINSON DA, RAIMI OG, BLAIR DE, HARRISON JR, LOCKHART DE, TORRIE LS, RUDA GF, WYATT PG, GILBERT IH, VAN AALTEN DM. 2015. N-myristoyltransferase is a cell wall target in *Aspergillus fumigatus*. ACS Chem Biol 10(6): 1425–1434.

FAWOLE OA, NDHLALA AR, AMOO SO, FINNIE JF, VAN STADEN J. 2009. Anti-inflammatory and phytochemical properties of twelve medicinal plants used for treating gastro-intestinal ailments in South Africa. J Ethnopharmacol 123(2): 237–243.

FELDMAN KS, SAHASRABUDHE K, LAWLOR MD, WILSON SL, LANG CH, SCHEUCHENZUBER WJ. 2001. *In vitro* and *in vivo* inhibition of LPS-stimulated tumor necrosis factor-α secretion by the gallotannin β-d-pentagalloylglucose. Bioorg Med Chem Lett 11(14): 1813–1815.

FERREIRA LG, DOS SANTOS RN, OLIVA G, ANDRICOPULO AD. 2015. Molecular docking and structure-based drug design strategies. Molecules 20(7): 13384–13421.

FILIMONOV DA, LAGUNIN AA, GLORIOZOVA TA,

RUDIK AV, DRUZHILOVSKII DS, POGODIN PV, POROIKOV VV. 2014. Prediction of the biological activity spectra of organic compounds using the Pass Online Web Resource. Chem Heterocyclic Comp 50(3): 444–457.

FLYNN E. 2008. Pharmacokinetic parameters. In: xPharm: the comprehensive pharmacology reference. Enna SJ, Bylund DB eds. Elsevier. p. 1–3.

GERLITS O, HO KY, CHENG X, BLUMENTHAL D, TAYLOR P, KOVALEVSKY A, RADIĆ Z. 2019. A new crystal form of human acetylcholinesterase for exploratory room-temperature crystallography studies. Chem Biol Interact 309(25): 108698.

GHOSE AK, VISWANADHAN VN, WENDOLOSKI JJ. 1999. A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery; 1. A qualitative and quantitative characterization of known drug databases. J Comb Chem 1(1): 55–68.

GSCHWEND DA, GOOD AC, KUNTZ ID. 1996. Molecular docking towards drug discovery. J Mol Recogn 9(2): 175–186.

GUERRERO-PERILLA C, BERNAL FA, COY-BARRERA ED. 2015. Molecular docking study of naturally occurring compounds as inhibitors of N-myristoyl transferase towards antifungal agents discovery. Rev Colomb Cienc Quím Farm 44(2): 162–178.

HESSE M. 2002. Alkaloids: nature's curse or blessing?. Wiley-VCH. p. 303–309

HIKMAT A. 2006. Kecenderungan populasi *Rafflesia zolingeriana* Kds. di Taman Nasional Meru Betiri, Jawa Timur. Media Konservasi 11(3): 105–108 (in Indonesian).

INDONESIAN MINISTRY OF FOREIGN AFFAIRS. 1990. UU RI no. 05/1990. Retrieved on 02 Jul 2022 from http://ksdae.menlhk.go.id/assets/uploads/1990-UU-05-Hayati-ekosistem.pdf

ISMAIL G. 1988. Conservation of the giant *Rafflesia* in Sabah, Malaysia. Trends Ecol Evol 3(12): 316–317.

ISTVAN ES, PALNITKAR M, BUCHANAN SK, DEISENHOFER J. 2000. Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. EMBO J 19(5): 819–830.

IWASHINA T, TANAKA N, AUNG MM, DEVKOTA HP, MIZUNO T. 2020. Phenolic compounds from parasitic *Sapria himalayana* f. *albovinosa* and *Sapria myanmarensis* (Rafflesiaceae) in Myanmar. Biochem Syst Ecol 93: 104179.

KANCHANAPOOM T, KAMEL MS, PICHEANSOONTHON C, LUECHA P, KASAI R, YAMASAKI K. 2007. Hydrolyzable tannins and phenylpropanoid from *Rafflesia kerrii* Meijer (Rafflesiaceae). J Nat Med 61: 478–479.

KISS AK, PIWOWARSKI JP. 2018. Ellagitannins, gallotannins, and their metabolites-the contribution to the anti-inflammatory effect of food products and medicinal plants. Curr Med Chem 25(37): 4946–4967.

LAGUNIN A, STEPANCHIKOVA A, FILIMONOV D, POROIKOV V. 2000. PASS: prediction of activity spectra for biologically active substances. Bioinformatics 16(8): 747–748.

LANZA AMD, MARTÍNEZ MJ, MATELLANO LF, CARRETERO CR, CASTILLO LV, SEN AMS, BENITO PB. 2001. Lignan and phenylpropanoid glycosides from *Phillyrea latifolia* and their *in vitro* anti-inflammatory activity. Plant Med 67(3): 219–223.

LASKOWSKI RA, SWINDELLS MB. 2011. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. J Chem Inf Model 51(10): 2778–2786.

LATGÉ JP. 1999. *Aspergillus fumigatus* and Aspergillosis. Clin Microbiol Rev 12: 310–350.

LIN LC, SHEN YC, WANG YH, LIOU KT, HOU YC, CHANG S, WANG WY, CHOU YC. 2006. The inhibitory effect of phenylpropanoid glycosides and iridoid glucosides on free radical production and β2 integrin expression in human leucocytes. J Pharm Pharmacol 58(1): 129–135.

LIPINSKI CA, LOMBARDO F, DOMINY BW, FEENEY PJ. 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Delivery Rev 23(1–3): 3–25.

MANAS ES, UNWALLA RJ, XU ZB, MALAMAS MS, MILLER CP, HARRIS HA, HSIAO C, AKOPIAN T, HUM WT, MALAKIAN K, WOLFROM S. 2004. Structure-based design of estrogen receptor-β selective ligands. J Am Chem Soc 126(46): 15106–15119.

MAT-SALLEH K. 1991. *Rafflesia*: Magnificent Flora of Sabah. Kota Kinabalu: Borneo Publishing Company.

MAYER AM. 1986. Polyphenol oxidases in plants-recent progress. Phytochemistry 26(1): 11–20.

MEIJER W, ELLIOTT S. 1990. Taxonomy, ecology, and conservation of *Rafflesia kerrii* Meijer in southern Thailand. Nat His Bull Siam Soc 38(2): 117–133.

MEIJER W. 1993. Rafflesiaceae. In: The Families and Genera of Vascular Plants Vol. 2. Kubitzki K, Rohwer JG and Bittrich V eds. Berlin: Springer. p. 557–563.

MOLINA J, MCLAUGHLIN W, WALLICK K, PEDALES R, MARIUS VM, TANDANG DN, DAMATAC A, STUHR N, PELL SK, LIM TM, NOVY A. 2017. *Ex situ* propagation of Philippine *Rafflesia* in the United States: challenges and prospects. Sibbaldia 15: 77–96.

MORIGUCHI I, HIRONO S, LIU Q, NAKAGOME I, MATSUSHITA Y. 1992. Simple method of calculating octanol/water partition coefficient. Chem Pharm Bull 40(1): 127–130.

MUEGGE I. 2006. PMF scoring revisited. J Med Chem 49(20): 5895–5902.

MURSIDAWATI S, HANDINI E. 2009. Biologi konservasi tumbuhan holoparasit: percobaan kultur *in vitro*. In: Proceedings of "Indonesian flora conservation in addressing the impact of global warming"; "Eka Karya" Botanical Garden, Bali, Indonesia. p. 158–162 (in Indonesian).

MURSIDAWATI S, IRAWATI. 2017. Biologi Konservasi *Rafflesia*. Jakarta: LIPI Press (in Indonesian).

MURSIDAWATI S, NGATARI N, IRAWATI I, CARDINAL S, KUSUMAWATI R. 2015. *Ex situ* conservation of *Rafflesia patma* Blume (Rafflesiaceae): an endangered emblematic parasitic species from Indonesia. Sibbaldia 13: 99–110.

MURSIDAWATI S, WICAKSONO A, TEIXEIRA DA SILVA JA. 2019. Development of the endophyte parasite, *Rafflesia patma* Blume, among host plant (*Tetrastigma leucostaphylum* (Dennst.) Alston) vascular cambium tissue. S Afr J Bot 123: 382–386.

NAIS J. 2001. *Rafflesia* of The World. Sabah Parks, Kota Kinabalu, Malaysia.

NIKOLOV LA, ENDRESS PK, SUGUMARAN M, SASIRAT S, VESSABUTR S, KRAMER EM, DAVIS CC. 2013. Developmental origins of the world's largest flowers, Rafflesiaceae. Proc Nat Acad Sci USA 110: 18578–18583.

NIKOLOV LA, TOMLINSON PB, MANICKAM S, ENDRESS PK, KRAMER EM, DAVIS CC. 2014. Holoparasitic Rafflesiaceae possess the most reduced endophytes and yet give rise to the world's largest flowers. Ann Bot 114(2): 233–242.

NORHAZLINI MZ, MAILINA J, SHALINI M, NORAZAH MA, ZULHAZMAN H. 2021. Total phenolic content, total flavonoid content, and antioxidant activity of ethanolic extract of *Rafflesia kerri* Meijer, Lojing Highlands, Peninsular Malaysia. IOP Conf Ser: Earth Environ Sci 842: 012062.

O'BOYLE NM, BANCK M, JAMES CA, MORLEY C, VANDERMEERSCH T, HUTCHISON GR. 2011. Open Babel: an open chemical toolbox. J Cheminform 3: 33.

OFFICIAL GAZETTE OF THE GOVERNMENT OF PHILIPPINES. 2001. Republic Act No. 9147. Retrieved on 02 Jul 2022 from https://www.officialgazette.gov.ph/2001/07/30/republic-act-no-9417

OGURO Y, MIYAMOTO N, OKADA K, TAKAGI T, IWATA H, AWAZU Y, MIKI H, HORI A, KAMIYAMA K, IMAMURA S. 2010. Design, synthesis, and evaluation of 5-methyl-4-phenoxy-5H-pyrrolo [3, 2-d] pyrimidine derivatives: novel VEGFR2 kinase inhibitors binding to inactive kinase conformation. Bioorg Med Chem 18(20): 7260–7273.

PARK E, KWON HY, JUNG JH, JUNG DB, JEONG A, CHEON J, KIM B, KIM SH. 2015. Inhibition of myeloid cell leukemia 1 and activation of caspases are critically involved in gallotannin-induced apoptosis in prostate cancer cells. Phytother Res 29(8): 1225–1236.

PENG FW, LIU DK, ZHANG QW, XU YG, SHI L. 2017. VEGFR-2 inhibitors and the therapeutic applications thereof: a patent review (2012–2016). Expert Opin Ther Pat 27(9): 987–1004.

PLANTS OF THE WORLD ONLINE. 2017. *Sapria*. Retrieved on 02 Jul 2022 from http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:14134-1

POWERS CN, SETZER WN. 2015. A molecular docking study of phytochemical estrogen mimics from dietary herbal supplements. In Silico Pharmacol 3(1): 4.

PUTTIPAN R, OKONOGI S. 2014. Antioxidant activity of *Rafflesia kerrii* flower extract. Drug Discov Ther 8(1): 18–24.

QUATTROCCHI UFLS. 2012. World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology. Boca Raton: CRC Press. p. 3197–3198.

REFAEI J, JONES EBG, SAKAYAROJ J, SANTHANAM J. 2011. Endophytic fungi from *Rafflesia cantleyi*: species diversity and antimicrobial activity. Mycosphere 2(4): 429–447.

ROUX DG, EVELYN SR. 1958. Condensed tannins. 2. Biogenesis of condensed tannins based on leucoanthocyanins. Biochem J 70(2): 344.

SABAH LAW NET. 2010 Wildlife Conservation Enactment 1997. Retrieved on 02 Jul 2022 from https://sagc.sabah.gov.my/sites/default/files/law/WildlifeConservation1997%28Regulations1998%29.pdf

SAHA P, TALUKDAR AD, NATH R, SARKER SD, NAHAR L, SAHU J, CHOUDHURY MD. 2019. Role of natural phenolics in hepatoprotection: a mechanistic review and analysis of regulatory network of associated genes. Front Pharmacol 10: 509.

SAHAKIAN B, JONES G, LEVY R, GRAY J, WARBURTON D. 1989. The effects of nicotine on attention, information processing, and short-term memory in patients with dementia of the Alzheimer type. Brit J Psychiat 154(6): 797–800.

SALEH Z, ZUHUD EA, SARI RK. 2015. Phytochemical screening and antioxidant activity of ethanolic extract of *Rhizanthes deceptor* (Rafflesiaceae) and its host *Tetrastigma papillosum*. Res J Med Plant 9(6): 293–299.

SARAWAK FORESTRY. 1998. Wild Life Protection Ordinance 1998. Retrieved on 02 Jul 2022 from https://www.sarawakforestry.com/pdf/laws/wildlife_protection_ordinance98_chap26.pdf

SCHÜTTELKOPF AW, VAN AALTEN DMF. 2004. PRODRG: a tool for high-throughput crystallography of protein–ligand complexes. Acta Cryst D 60(8): 1355–1363.

SEO EJ, KUETE V, KADIOGLU O, KRUSCHE B, SCHRÖDER S, GRETEN HJ, AREND J, LEE IS, EFFERTH T. 2013. Antiangiogenic activity and pharmacogenomics of medicinal plants from traditional Korean medicine. Evid Based Complement Alternat Med [131306].

SHARGEL L, YU AB. 2015. Applied Biopharmaceutics & Pharmacokinetics, 7th ed. New York: McGraw-Hill.

SOFIYANTI N, WAHIBAH NN, PURWANTO D, SYAHPUTRA ED, MAT-SALLEH K. 2008. Alkaloid and phenolic compounds of *Rafflesia hasseltii* Suringar and its host *Tetrastigma leucostaphylum* (Dennst.) Alston ex Mabb. in Bukit Tigapuluh National Park, Riau: A preliminary study. Biodiversitas J Biol Div 9(1): 17–20.

SOGABE S, MASUBUCHI M, SAKATA K, FUKAMI TA, MORIKAMI K, SHIRATORI Y, EBIIKE H, KAWASAKI, K, AOKI Y, SHIMMA N, D'ARCY A. 2002. Crystal structures of *Candida albicans* N-myristoyltransferase with two distinct inhibitors. Chem Biol 9(10): 1119–1128.

SUKAMTO LA. 2001. Upaya menumbuhkan *Rafflesia arnoldii* Secara *in vitro*. In: Prosiding Nasional Puspa Langka. Bogor. p. 31–34 (in Indonesian).

SUKAMTO LA, MUJIONO M. 2010. *In vitro* culture of holoparasite *Rafflesia arnoldii* R. Brown. Buletin Kebun Raya 13: 79–85.

SUNG H, FERLAY J, SIEGEL RL, LAVERSANNE M, SOERJOMATARAM I, JEMAL A, BRAY F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: Cancer J Clin 71(3): 209–249.

SYAIFUDDIN, ANDRIANI S, SURYANTO E, NISA LS, WARDANA AZ, KURNIAWAN NMA. 2018. Kajian pemanfaatan tumbuhan obat sebagai obat tradisional di Kabupaten Hulu Sungai Tengah Propinsi Kalimantan Selatan. Prosiding Seminar Nasional Teknologi Hasil Hutan. p. 317–325 (in Indonesian).

TROTT O, OLSON AJ. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comp Chem 31(2): 455–461.

UN ENVIRONMENT PROGRAMME. 2021. Protection of Wildlife Act 1972 (No. 76 of 1972). Retrieved on 29 Nov 2021 from https://leap.unep.org/countries/my/national-legislation/protection-wildlife-act-1972-no-76-1972

VAN DER VRIES E, COLLINS PJ, VACHIERI SG, XIONG X, LIU J, WALKER PA, HAIRE LF, HAY AJ, SCHUTTEN M, OSTERHAUS AD, MARTIN SR. 2012. H1N1 2009 pandemic influenza virus: resistance of the I223R neuraminidase mutant explained by kinetic and structural analysis. PLoS Pathogens 8: e1002914.

VEBER DF, JOHNSON SR, CHENG HY, SMITH BR, WARD KW, KOPPLE KD. 2002. Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 45(12): 2615–2623.

VIDYA SM, KRISHNA V, MANJUNATHA BK, BHARATH BR, RAJESH KP, MANJUNATHA H, MANKANI KL. 2012. Wound healing phytoconstituents from seed kernel of *Entada pursaetha* DC. and their molecular docking studies with glycogen synthase kinase 3-β. Med Chem Res 21: 3195–3203.

WANG X, BOVE AM, SIMONE G, MA B. 2020. Molecular bases of VEGFR-2-mediated physiological function and pathological role. Front Cell Dev Biol 8: 599281.

WANG Y, PENG C, WANG G, XU Z, LUO Y, WANG J, ZHU W. 2019. Exploring binding mechanisms of VEGFR2 with three drugs lenvatinib, sorafenib, and sunitinib by molecular dynamics simulation and free energy calculation. Chem Biol Drug Design 93(5): 934–948.

WANGCHUK P, KELLER PA, PYNE SG, TAWEECHOTIPATR M, TONSOMBOON A, RATTANAJAK R, KAMCHONWONGPAISAN S. 2011. Evaluation of an ethnopharmacologically selected Bhutanese medicinal

plants for their major classes of phytochemicals and biological activities. J Ethnopharmacol 137(1): 730–742.

WASS MN, KELLEY LA, STERNBERG MJ. 2010. 3DLigandSite: predicting ligand-binding sites using similar structures. Nucleic Acid Res 38(Suppl. 2) W469–473.

WIART C, MOGANA S, KHALIFAH S, MAHANB M, ISMAIL S. BUCKLE M, NARAYANA AK, SULAIMAN M. 2004. Antimicrobial screening of plants used for traditional medicine in the state of Perak, Peninsular Malaysia. Fitoterapia 75(1): 68–73.

WICAKSONO A, CRISTY GP, RAIHANDHANY R, MURSIDAWATI S, TEIXEIRA DA SILVA JA, SUSATYA A. 2021. *Rhizanthes*, the forgotten relative of *Rafflesia* in the Rafflesiaceae. Bot Rev 18: 1–4.

WICAKSONO A, MURSIDAWATI S, SUKAMTO LA, TEIXEIRA DA SILVA JA. 2016. *Rafflesia* spp.: propagation and conservation. Planta 244: 289–296.

WICAKSONO A, MUTTAQIN M. 2020. Mencoba Menguak Misteri *Rafflesia* dan Tumbuhan Parasit Indonesia di Masa Pandemi COVID-19. In: Bunga Rampai Forum Peneliti Muda Indonesia 2020: Tetap Berkarya dalam Masa Pandemi COVID-19. Bandung: ITB Press. p. 81–94 (in Indonesian).

WICAKSONO A, TEIXEIRA DA SILVA JA. 2015. Attempted callus induction of holoparasite *Rafflesia patma* Blume using primordial flower bud tissue. Nus Biosci 7(2): 96–101.

WICAKSONO A, TEIXEIRA DA SILVA JA, MURSIDAWATI S. 2017. Dispersal of *Rafflesia patma* Blume endophyte in grafted host plant (*Tetrastigma leucostaphylum* (Dennst.) Alston). J Plant Dev 24: 145–150.

WU-YUAN CD, CHEN CY, WU RT. 1988. Gallotannins inhibit growth, water-insoluble glucan synthesis, and aggregation of mutant streptococci. J Dent Res 67(1): 51–55.

YE YC, ZHAO XL, ZHANG SY. 2015. Use of atorvastatin in lipid disorders and cardiovascular disease in Chinese patients. Chin Med J 128(2): 259–266.

ZAMAN MQ. 2009. Etnobotani tumbuhan obat di Kabupaten Pamekasan Madura Provinsi Jawa Timur [undergraduate thesis]. Universitas Islam Negeri Maulana Malik Ibrahim Malang, Malang.

ZHANG DL, GU LJ, LIU L, WANG CY, SUN BS, LI Z, SUNG CK. 2008. Effect of Wnt signaling pathway on wound healing. Biochem Biophys Res Commun 378(2): 149–151.

ZHAO X, SUN H, HOU A, ZHAO Q, WEI T, XIN W. 2005. Antioxidant properties of two gallotannins isolated from the leaves of *Pistacia weinmannifolia*. Biochim Biophys Act – General Subjects 1725(1): 103–110.

ZUHUD EAM, HERNIDIAH N, HIKMAT A. 1999. Pelestarian *Rafflesia hasseltii* Suringar di Taman Nasional Bukit Tigapuluh Riau-Jambi. Media Konservasi 6(1): 23–26 (in Indonesian).

ZULKFFLE MA, OSMAN NS, YUSOFF Z, KAMAL ML, TAJAM J. 2014. Identification of alkaloid compound and antioxidant activity of *Rafflesia cantley*i and its host, *Tetrastigma tuberculatum*. Open Conf Proc J 5: 18–20.