Absorption, distribution, metabolism, excretion, and toxicity evaluation of Papua red fruit flavonoids through a computational study

M M Suprijono¹,², H Sujuti³, D Kurnia⁴ and S B Widjanarko⁵

¹ Department of Food Technology, Agricultural Technology Faculty, Widya Mandala Catholic University Surabaya, Indonesia.
² Doctoral Program of Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia
³ Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
⁴ Department of Chemistry, Faculty of Mathematics and Natural Science, Padjajaran University, Bandung, Indonesia
⁵ Department of Food Science and Technology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

E-mail: maria-matoetina@ukwms.ac.id, m.matoetina@gmail.com

Abstract. Red Fruit (RF) (Pandanus conoideus Lam.) was used as traditional medicine for Papuans and consumed as a daily meal. RF was proved as a source of bioactive antioxidant and anticancer, grace on their flavonoids. There was a few researches that focuses on the metabolism and toxicity of RF, but the mechanism of metabolism and toxicity of the flavonoids present in the RF is unclear. This research aims to evaluate the absorption, distribution, metabolism, excretion, and toxicity of RF flavonoids through computational study, sharpening their potency as bioactive in functional food. Flavonoids in RF extracts were identified using LCMS and or obtained from secondary data. The chemical structure of the flavonoids was redrawn to get the canonical of the molecular graph. Absorption, distribution, metabolism, and excretion were predicted using SWISS ADME, OSIRIS Property Explorer, and hERG-Pred. Those were based on the physicochemical properties, pharmacokinetic, BOILED-EGG test, whereas the toxicology based on the potency as a toxicant, P-gp substrate, hERG blocker, and CYP450 inhibitor. Quercetin, Taxifolin, and Quercetin 3-Glucoside were identified using the methanol and ethyl acetate extract. Quercetin, Taxifolin, 3,4,5-trihydroxy-7,3-dimethoxyflavone/TDF, 4',6,6',8-tetrahydroksi-3-metoksi-flavon/TMF, and Quercetin3-Methyl-Ether/QME fulfilled the RO5 parameters; they were higher in water solubility, gastrointestinal absorption, and bioavailability. All were not P-gp substrate and hERG blocker, but some of them were CYP450 inhibitors. Only TMF, QME, Taxifolin3-O-α-Arabinopiranose/TAP, and Quercetin3-O-Glucose/QOG that consistently had no risk as toxic compounds. RF flavonoid showed high potency as bioactive. Most of the flavonoid had no toxicity risk. Generally, RF flavonoids were categorised as safe.

1. Introduction
Nowadays, there is a large number of molecular structures of chemicals evaluated to select which chemicals gave the best chance to become an effective bioactive in functional food or drug in medical
needs. The molecule must show high bioactivity but also low toxicity. In point of view of availability and toxicity, the absorption, distribution, metabolism, excretion, and toxicity are the parameter that has to be measured and evaluated.

The assessment of toxicology risk usually done with animal experimentation, but time and financial consume [1,2]. In silico pharmacokinetic and pharmacological studies were performed based on absorption, distribution, metabolism, and excretion toxicity (abbreviated as ADMET) [3]. The method is a valid alternative to the experimental, especially at the initial steps of research [2]. In silico toxicology are familiar used in drug research, but rarely in functional food research. ADMET analysis must be made along with bioactive analysis.

Red Fruit (Pandanus conoideus Lam.) is an indigenous plant from Papua-Indonesia. Traditionally, the fruit was used as food and also a health supplement. Papua Red Fruits were potentially used as nutrient and pigment source, antioxidant, and anticancer [4-8]. Research done on Red Fruit has not covered bioactive compound responsible for the activity (antioxidant and anticancer). As far as our concern, there is little research done for risk toxicity of compound found in this fruit. RF Toxicity analysis was done in terms of cytotoxicity to cancer cells [4,8]. Although the LD50 of RF oil was 5 ml/200 g body weight of the rat, it considers as pseudo [9] because of no animal dead in the experiment of RF oil toxicity [9,10]. The reason for this condition is unclear.

The objective of this research was to evaluate the ADMET of flavonoids found in Papua Red Fruit (Pandanus conoideus Lam.) as an initial stage for utilisation of the fruit as a functional food.

2. Materials and Method

2.1. Plant materials and chemicals

The Red Fruit/RF (Pandanus conoideus Lam.) used as a sample was classified as the Short-Red cultivar, known in local name as Monsor. The plant was cultivated in the Garden Laboratory of Papua State University, Manokwari, West Papua, Indonesia. The fruit was harvested on the period of November 2016. Species identification of the plant was made by Molecular Genetic Laboratory, Fishery Faculty, Papua State University, Manokwari, West Papua, Indonesia. All chemicals for extraction and partition (methanol, hexane, and ethyl acetate) were analytical grade, and for LC-MS/MS analysis (methanol, acetonitrile, formic acid) were HPLC grade. Gallic acid/GA (SigmaAldrich 27645) and quercetin/Q (Sigma-Aldrich Q4951) was used as a standard for total phenolic and total flavonoid content analysis, respectively.

2.2. RF extraction

The grains (drupe) of RF were separated from the fruit (cepallum), then macerated in methanol absolute for six days at room temperature. The filtrate of macerate was evaporated at 40ºC, which called Methanol Extract/ME. A part of the ME was partitioned using hexane:water then ethyl acetic to get Ethyl Acetic Extract/EE [5,11].

2.3. Total phenolic and total flavonoid content determination

This analysis used the spectrophotometry method [5,12]. TPC was calculated and then expressed as mg Gallic Acid equivalents (GAE) per gram of extract (mg/g). The total flavonoid content then was stated as mg Quercetin equivalents (QE) per gram of extract (mg/g).

2.4. LC-MS analysis

2.4.1. Sample and standard preparation

RF methanol extract (13-40 mg) was dissolved in 1 ml of methanol. It was sonicated for 5 minutes then centrifuged at 5000 rpm for 5 minutes. The solution was passed through membrane filter 0.22μm.

2.4.2. UHPLC analysis

The system is Liquid Chromatography-High Resolution Mass Spectrometry (Thermo Scientific Dionex Ultimate 3000 RSLCnano), which completed with a nano pump with microflow meter,
vacuum degasser, and thermostatic autosampler. Separations were done on Hypersil GOLD aQ 50 x 1 mm x 1.9 μ particle size. The temperature was set at 30°C for the column. The mobile phase was 0.1% formic acid in water (v/v) (A) and 0.1% formic acid in methanol (v/v) (B). The gradient elution was performed by 60% (B) for 0 to 2.00 minutes and continued 2.00 to 15.00 minutes, at a flow rate of 40 μL/min. The injection volume was 10 μL.

2.4.3. MS analysis
The Mass Spectrophotometer used Electrospray Ionization (ESI) in negative mode, with switch the polarity. It is high-resolution MS. Scanning mode was performed by PRM with MS2 at 17,500 resolution; maximum IT at 100 ms, isolation window at 1.0 m/z, and (N)CE / stepped was set at 30, 45, 70. The analysis was run for 15 minutes. Data processing software was Compound Discoverer with mzCloud MS/MS Library.

### Table 1. The inclusion mass range setting of LCMS/MS

| Mass [m/z] | Formula [M] | Species | CS [z] | Polarity | Comment |
|-----------|-------------|---------|--------|----------|---------|
| 353.08781 | C₁₆H₁₈O₉ | -H | 1 | Negative | Chlorogenic Acid |
| 301.03538 | C₁₅H₁₀O₇ | -H | 1 | Negative | Quercetin |
| 463.08820 | C₂₁H₁₂O₁₂ | -H | 1 | Negative | Quercetin 3-beta-D-Glucoside |
| 305.06558 | C₁₅H₁₂O₇ | +H | 1 | Positive | Taxifolin |
| 355.10236 | C₁₆H₁₈O₉ | +H | 1 | Positive | Chlorogenic acid |
| 303.04993 | C₁₅H₁₀O₇ | +H | 1 | Positive | Quercetin |
| 465.10275 | C₂₁H₂₀O₁₂ | +H | 1 | Positive | Quercetin 3-beta-D-Glucoside |
| 303.05103 | C₁₅H₁₂O₇ | -H | 1 | Negative | Taxifolin |

2.5. Computational analysis
2.5.1. Ligands preparation.
Ligands used in this research are The RF flavonoids identified by LCMS and six of the RF flavonoid Isolated by Research Laboratory of Chemistry Department FMIPA Padjajaran University Bandung/UNPAD, Indonesia with permission of Dr. Dikdik Kurnia, M.Sc. (Head of Laboratory). The ligands are Quercetin, Taxifolin, Quercetin 3'-Glucose (Q3G), 3,4',5-trihydroxy-7,3'-dimethoxyflavan (TDF), 4',6,6',8-tetrahydroxy-3-methoxy-flavon (TMF), Taxifolin 3-O-alpha-arabinopyranose (TAP), Quercetin 3-O-glucose (QOG), and Quercetin 3-methyl-ether (QME). Chemical structures of TDF, TMF, TAP, QOG, and QME are redrawn using PubChem Structure Drawing: Sketcher V2.4 - Mozilla Firefox, whereas the structure of Quercetin, Taxifolin, and Q3G are obtained from PubChem compound database (CID 5280343, 439533, and 5280804). The canonical of a simplified molecular-input line-entry system (SMILE) of each flavonoid is used for the computational analysis.

2.5.2. Absorption, distribution, metabolism, and excretion (ADME) prediction
ADME (physicochemical, absorption, and bioavailability) was predicted using Swiss ADME [2]. The absorption was calculated based on the formula below:

$$\%\text{ABS} = 109 - (0.345 \times \text{TPSA})$$  \(\text{(1)}\)

Gastrointestinal (GI) absorption and Blood-Brain Barrier (BBB) permeant were determined according to the position on white or yolk of BOILED-EGG [2,13].
2.5.3. Toxicity risk prediction
Toxicity risk consists of mutagenic, tumorigenic, irritant, and reproductive effects were predicted using the OSIRIS Property Explorer program, download from https://www.organic-chemistry.org/prog/peo/ [14]. The prediction of P-gp substrate and CYP450 inhibition was made using SwissADME [2]. Whereas the hERG blocker was analysed using Pred-hERG 4.1 (http://predherg.labmol.com.br) [15].

3. Results and Discussion
3.1. Total phenolic content and flavonoid identification
RF was known as a source of phenolic. The short-red cultivar RF in this research was found contained total phenolic 24.02 and 48.97 mg GAE/g or 2.4 and 4.9 % for methanol and ethyl acetate extract, whereas total flavonoid 4.51 and 14.05 mg QE/g or 0.45 and 1.41 % for methanol and ethyl acetate extract. Those are lower than the one found in other RF [5,12]. However, the same pattern that ethyl acetate extract had higher total phenolic and flavonoid than methanol extract. The difference in RF variety or cultivar and district where it grows may cause the difference in physicochemical properties and bioactive profile [6,7].

Table 2. Phenolic detected in methanol extract of Pandanus conoideus Lam. using LCMS/MS

| No | RT (minute) | measured mass (m/z) | ms2 (m/z) | Identified Compounds       |
|----|-------------|---------------------|-----------|---------------------------|
| 1  | 0.88        | 354.09              | 353.09    | Chlorogenic Acid          |
| 2  | 0.94        | 304.06              | 305.06    | Taxifolin                 |
| 3  | 0.97        | 464.09              | 463.09    | Quercetin-3β-D-Glucoside  |
| 4  | 3.79        | 302.04              | 301.04    | Quercetin                 |

Note: RT means Retention Time

Table 2 showed that quercetin, taxifolin, and quercetin-3-glucoside were flavonoid-detected in RF methanol extract. It seems these three flavonoids are dominant since they were found not only in this short-red cultivar but also in long-red cultivar RF [16]. Based on the substitution of molecular structures, they all are classified as flavonols. The three flavonoids in this RF and six others in long-red cultivar found by the UNPAD research team are 15 carbon-skeleton that possess two benzene rings (B and A-ring) joined by heterocyclic C-ring [16-18]. They have a hydroxyl group at 3’ position of C ring, but methylated for TMF and QME, or glycosylated for Q3G, TAP, and QOG. The structures confirmed that RF flavonoids were quercetin and or taxifolin derivatives. Taxifolin is a dihydroflavonol, flavonoid compound class that is a subclass of flavonols [18].

3.2. ADME evaluation
This evaluation is a vital step in the potential prediction of the particular compound as bioactive. The use of the Lipinski Rule of 5 (RO5) strategy can be used for the prediction of the bioactive compound in functional food [3]. Rule of 5 parameters is molecular weight (MW) ≤ 500 g/mol, partition coefficient (LogP) ≤ 5, H-bond acceptors (HBA) ≤ 10, H-bond donors (HBD) ≤ 5 [19]. The RO5 strategy can be used for the prediction of the bioactive compound in functional food [3]. These parameters are correlated to acceptable aqueous solubility and intestinal permeability. Those are parameters for oral bioavailability prediction [20], but Kauthale et al. (2017) added NRB <10, and H/C ratio <1 and molecular percent of absorption 100% [3].

Table 3 and 4 showed that only five from eight flavonoids that fulfilled the parameters; those are Quercetin, Taxifolin, TDF, TMF, and QME. Those are good in oral-bioavailability. It seems that the high polarity (TPSA and CLogP value) of Q3G, TAP, and QOG that make them had very low absorption, and bioavailability score then has to be out of RO5 parameters. Quercetin, Taxifolin, TDF,
TMF, and QME were located in white of Egan- or Boiled-Egg that determined the high permeability through the gastrointestinal tract [2,13], showing their high absorption.

### Table 3. Physicochemical properties of toxicity red fruit flavonoids

| Compound Name | Physicochemical Properties | Lipophilicity | Water Solubility |
|---------------|----------------------------|---------------|------------------|
|               | MW (g/mol) | Fraction Sp3 | N.RB | N.HBAs | N.HBDs | MR | TPSA (Å²) | Consensus LogP<sub>ow</sub> | Class |
| Quercetin     | 301.04     | 0            | 1    | 7      | 5      | 78.03 | 131.36 | 1.23 | Soluble |
| Taxifolin     | 305.06     | 0.13         | 1    | 7      | 5      | 74.76 | 127.45 | 0.63 | Soluble |
| Q3G           | 463.09     | 0.29         | 4    | 12     | 8      | 110.16 | 210.51 | -0.25 | Soluble |
| TDF           | 329.28     | 0.12         | 3    | 7      | 3      | 85.81 | 109.36 | 1.98 | Moderately soluble |
| TMF           | 316.26     | 0.06         | 2    | 7      | 4      | 82.50 | 120.36 | 1.75 | Soluble |
| TAP           | 436.37     | 0.35         | 3    | 11     | 7      | 101.17 | 186.37 | -0.50 | Soluble |
| QOG           | 464.38     | 0.29         | 4    | 12     | 8      | 110.16 | 210.51 | -0.25 | Soluble |
| QME           | 316.26     | 0.06         | 2    | 7      | 4      | 82.50 | 120.36 | 1.75 | Soluble |

Notes: MW: Molecular Weight, LogP<sub>ow</sub>; average prediction, N.RB: Number of Rotatable Bonds, N.HBAs: Number of H-bond Acceptors, N.HBDs: Number of H-bond Donors, MR: Molar Refractivity, TPSA: Topological Polar Surface Area

### Table 4. The absorption and bioavailability prediction of red fruit flavonoids.

| Compound Name | Absorption | GI Absorption# | BBB Permeant# | Bioavailability Score |
|---------------|------------|----------------|---------------|-----------------------|
| Quercetin     | 63.68      | High           | No            | 0.55                  |
| Taxifolin     | 65.03      | High           | No            | 0.55                  |
| Q3G           | 36.37      | Low            | No            | 0.17                  |
| TDF           | 71.27      | High           | No            | 0.55                  |
| TMF           | 67.48      | High           | No            | 0.55                  |
| TAP           | 44.70      | Low            | No            | 0.17                  |
| QOG           | 36.37      | Low            | No            | 0.17                  |
| QME           | 67.48      | High           | No            | 0.55                  |

Notes: ABS: Absorption; GI: Gastrointestinal; BBB: Blood-Brain Barrier

### 3.3. Toxicity evaluation

From eight flavonoids, TMF, TAP, QOG, and QME consistently have no risk as toxic compounds (Table 4). Our concern was on taxifolin, which has a high risk of toxicity as a mutagenic, tumorigenic, irritant, and reproductive effect. The experiment using RF extract of yellow cultivar showed that there was no influence in the percentage of the living fetus, but there was lordosis and ossification disturbance in the fetus [21]. The toxicity of the compound can be determined by the structure of its toxic functional group. The present of the C3-OH group, C-6 keto group, and or catechol group in B-ring in flavonoid may contribute to its mutagenic activity, whereas C7-OH may lead to genotoxicity [22]. Based on the substitution of the flavonoid [16], the present of C3-OH, and C7-OH in quercetin and taxifolin, C3-OH in TDF, and C7-OH in Q3G may contribute to their mutagenicity.

Table 5 shows that all RF flavonoid was not a P-gp substrate. P-gp is an essential protein of membrane cell that has to pump out foreign substances from the cell then avoid the risk of toxicity [3]. P-gp is the front line of body defence [23]. Those flavonoids will be detected by the cell as safe or non-toxic compounds, then enter the cells efficiently and play their function intracellularly. RF Flavonoids did not blockade the hERG K+ channel with the level of confidence 70% that means safe for cardiac cells. It is vital for cardiac activity, which controls the conduction of impulse. The blockage of the channel will lead to cardiac shock and lethal arrhythmic attack [3,15]. All RF flavonoid tested did not inhibit the activity of the CYP2C19 enzyme, but Quercetin, TDF, TMF, and QME inhibited CYP1A2, CYP2D6, and CYP3A4 enzyme activity. These make quercetin, TDF, TMF,
and QME were metabolized in the liver. The methylation in TDF, TMF, and QME influenced the inhibition, but glycosylation in Q3G, TAP, and QOG had no favourable for the CYP450 inhibition.

**Table 5. The risk of mutagenicity, tumorigenicity, irritant potency and reproductive effect of red fruit flavonoids**

| Compound Name | OSIRIS Prediction |
|---------------|-------------------|
|               | ME | TE | IE | RE |
| Quercetin     | +++| +++| -  | -  |
| Taxifolin     | +++| +++| +++| +++|
| Q3G           | +++| +++| -  | -  |
| TDF           | +++| -  | -  | -  |
| TMF           | -  | -  | -  | -  |
| TAP           | -  | -  | -  | -  |
| QOG           | -  | -  | -  | -  |
| QME           | -  | -  | -  | -  |

Notes: ME: Mutagenic Effect, TE: Tumorigenic Effect, IE: Irritant Effect, RE: Reproductive Effect, DL: Drug Likeness, DS: Drug Score. Potential toxicants: +: Low Risk, ++: Medium Risk, +++: High Risk, -: Non-Toxic

**Table 6. Risk toxicity of red fruit flavonoids**

| Compound Name | P-gp substrate | Pred-hERG result | CYP450 Inhibition |
|---------------|----------------|------------------|-------------------|
|               |                |                  | CYP1A2 | CYP2C19 | CYP2C9 | CYP2D6 | CYP3A4 |
| Quercetin     | No             | NB               | Yes    | No      | No     | Yes    | Yes    |
| Taxifolin     | No             | NB               | No     | No      | No     | No     | No     |
| Q3G           | No             | NB               | No     | No      | No     | No     | No     |
| TDF           | No             | NB               | Yes    | No      | Yes    | No     | Yes    |
| TMF           | No             | NB               | No     | No      | Yes    | Yes    | Yes    |
| TAP           | No             | NB               | No     | No      | No     | No     | No     |
| QOG           | No             | NB               | No     | No      | No     | No     | No     |
| QME           | No             | NB               | Yes    | No      | No     | Yes    | Yes    |

Notes: P-gp: Permeability Glycoprotein, hERG: Human ether-a-go-go related gene, NB: Non-Blocker, CYP: cytochrome

CYP450 is a family of the enzyme in the liver that catalyzed the biotransformation of most drugs, exogenous chemicals, or lipophilic xenobiotics [23,24]. CYP enzymes usually transform exogenous chemicals into less toxic and more hydrophilic compounds. Reactive intermediates as a result of CYP450 interaction with exogenous chemicals metabolizing systems may induce toxicity and carcinogenicity. If the reaction cannot be catalyzed with enzyme systems, the compound will not cause toxicity [23,25]. Inhibition of CYP enzymes may lead to toxic or another form of the unwanted effect of exogenous substances in the body, because of the minimal clearance and accumulation of the compounds or their metabolites [2]. SGPT and SGOT enzymes play as a detector for function disturbance of the liver. Water and oil fractions of RF ethanol extract at the dose range 500-1500 mg/Kg weight did not influence significantly on SGPT/SGOT rat Sprague Dawley [26]. Those mean even though Quercetin, TDF, TMF, and QME can pass the first line of defense and may do not disturb liver function, but they still have a chance to be toxic in the body because of their low hydrophilicity.

4. **Conclusions**

ADMET analysis needs to be done in the early stage of functional food research with bioactive compounds. RF was well known as a source of functional compounds thanks to the bioactive flavonoid. RF flavonoids were safe for cardiac cells. Quercetin, Taxifolin, Q3G, and TDF might cause a mutagenic effect, then had a risk as a toxic compound, whereas TMF, TAP, QOG, and QME showed
no-risk of toxic. Most of the flavonoids could be metabolized in the liver that reduces the risk of toxicity. It generally means RF flavonoids were safe for humans.

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