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Ethanolic extract of pomegranate (*Punica granatum* L) peel: acute toxicity tests on zebrafish (*Danio rerio*) embryos and its toxicity prediction by *in silico*

Indra Wibowo[1*], Kurnia Permadi[2], Rika Hartati[2], Sophi Damayanti[2*]

[1]School of Life Science and Technology, Bandung Institute of Technology, Jalan Ganesa No.10, Bandung, Indonesia.
[2]School of Pharmacy, Bandung Institute of Technology, Jalan Ganesa No.10, Bandung, Indonesia.

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ABSTRACT

Antibiotic resistance causes many serious health problems that have emerged both in developed and developing countries. One of many examples of the antibiotic problem is resistance to *Mycobacterium tuberculosis* in tuberculosis standard therapy. Various treatments have been done to overcome *Mycobacterium tuberculosis* resistance. Therefore, developing new compound or supplement from a natural product is widely explored. Pomegranate fruit (*Punica granatum* L) has been used traditionally as an antimicrobial agent. However, its toxicity should be investigated prior to development. This research was aimed to determine LC_{50} of the ethanolic extract and to predict the toxicity of the compound *in vivo* and *in silico*, respectively. The fish embryo acute toxicity test was done by using zebrafish (*Danio rerio*) embryos as subject to obtained safety characteristics of extract. Toxicities of compounds in pomegranate were predicted using software ADMET Predictor 7.1. Pomegranate peel ethanol extract revealed LC_{50} of 196,037 ± 9,2 µg/mL. Based on OECD aquatic toxicity classification, pomegranate peel ethanol extract was classified as safe. Toxicity prediction showed that brevifolin was the safest substance among the other substances that are contained in ethanol extract of pomegranate peel. Further research based on this result may lead to improving further natural drug development to overcome the resistance of antibiotic in tuberculosis.

INTRODUCTION

Ministry of Health Indonesia in 2011 stated that tuberculosis is one of the infectious diseases in Indonesia caused by *Mycobacterium tuberculosis (MTB)*. Each year, tuberculosis is capable to cause about two million deaths worldwide with 5.8% of these patients being Indonesians. These bacteria can hide in the human cells such as macrophage for staying dormant but can be activated if the body's defense system decreases (Clifton *et al.*, 2009).

As reported by Dheda *et al.* (2010), compounds from natural ingredients are the focus of antibacterial compound studies, especially for MTB to overcome rapid incidents of anti-bacterial resistance. Therefore, the discovery of new compounds as a substitute for existing drug compounds is urgently needed. Alpha mangostin was reported to be used for study its effect in MTB multidrug-resistant (Nugrahaeni *et al.*, 2016). In addition, other natural originated ursolic acid has been investigated as a future antimycobacterial drug (Pitaloka *et al.*, 2017).

However, due to a large amount of chemical content contained in the skin of pomegranates, it should be investigated which compound has antituberculosis activity. In addition, toxicity testing also needs to be done to ensure the safety level of pomegranate skin extract.

Toxicity tests can be done either *in silico* or *in vivo*. *In silico* prediction of toxicity based on the theory of computational chemistry whereas *in vivo* toxicity test by acute toxicity was performed in zebrafish embryo (Mc.Rae and Peterson, 2015).

[*]*Corresponding Author*
Indra Wibowo, School of Life Science and Technology, Bandung Institute of Technology, Jalan Ganesa No.10, Bandung, Indonesia. E-mail: indra @ sith.itb.ac.id; Sophi Damayanti, School of Pharmacy, Bandung Institute of Technology, Jalan Ganesa No.10, Bandung, Indonesia. E-mail: sophi.damayanti @ fa.itb.ac.id

Therefore, the aim of this study was to determine LC_{50} of the extract zebrafish (*Danio rerio*) embryo and to predict the toxicity of the compound in various parameters.

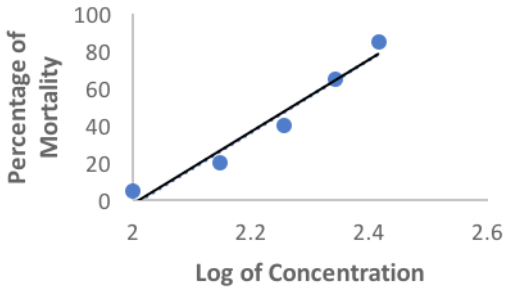

Fig. 1: The curve between log concentrations vs percent mortality of pomegranate skin ethanol extracts on zebrafish embryos. $y = 193.98x - 389.91$. $R^2 = 0.95546$.

MATERIALS AND METHODS

Materials

Ethanolic extract pomenagrate peel (*Punica granatum* L) from Pharmaceutical Biology School of Pharmacy Laboratory, *zebrafish* (*Danio rerio*) *wild-type* Bogor, DMSO, TLC silica gel GF_{254}.

Software

ADMET Predictor 7.1, dan Minitab v.17.

Methods

Pomegranate crude drug was standardized and characterized in parameters of water-soluble and ethanol content, moisture content, loss on drying, total ash content, water-soluble ash content, and acid soluble ash content. The ethanolic extract was characterized by its phytochemical screening.

The ethanol extract of pomegranate was then tested against zebrafish embryos with a test time of 96 hours. The test was carried out according to Organization for Economic Co-operation and Development (OECD) Test No. 236, 2013.

Adult zebrafish were obtained from local pet shop located in West Java Indonesia. These fish showed wild-type characteristics as described by Singh and Nüsslein-Volhard (2015). All fish were maintained and reared according to standard procedures, as well as fish breeding for egg production and collection (Kimmel *et al.*, 1995; Nagel 2002). For the purpose of zebrafish embryo toxicity test (FET), the procedures were adapted from several standard methods (Lammer *et al.,* 2009, OECD, 2013, Truong *et al.,* 2011, Busquet *et al.,* 2014). In short, freshly laid embryos were selected only that showed normal development until 6 hpf. The normally developed embryos were transferred to 24-well plates and incubated with ethanolic extract of pomegranate. The plate then incubated in an incubator 27.0 ± 1.0°C and observed every day for 96 hpf. The output observed during the embryo toxicity test were coagulation, somite disruption, non-detachment of the tail, and lack of heartbeat (OECD, 2013).

Categorization of embryo test was made reliable if embryo mortality of the negative control (≤10%) and the 3,4-dichloroaniline-treated embryos as a positive control (20-80%). All the tests were performed in triplicates. LC_{50} was determined using Minitab V.17. program.

All statistical analysis for zebrafish toxicity assay were conducted using Probit analysis with Weibull distribution/model. For determining whether the Weibull model was formed according to available data, another statistical analysis called Pearson's goodness-of-fit test was performed. The data were presented as means ± SD with the threshold set to $p > 0.05$.

Toxicity prediction was done using ADMET Predictor 7.1 program. Tested compounds were punicalin, gallocatechin, corilagin, gallic acid, ellagic acid, caffeic acid, luteolin, quercetin, brevifolin, punicalagin, hexahydroxy diphenoil acid, kaempferol, punicate acid (S1-S13).

RESULT AND DISCUSSION

Phytochemical screening was performed to determine the chemical content of pomegranate skin. The screening was performed both for crude drug and extract. The results of phytochemical screening data are listed in Table 1. Tanine, saponine, quinon, steroid/triterpenoid, and flavonoid are identified in both crude drug and extract.

Table 1: Phytochemical Screening Results of Crude Drug and Extracts.

Compound	Crude Drug	Ethanolic extract
Tanine	+	+
Saponine	+	+
Quinon	+	+
Steroid/triterpenoid	+	+
Flavonoid	+	+
Alkaloid	–	–

To ensure the quality of test materials used, standardization and characterization of crude drug and extracts were used. The results of standardization are shown in Table 2. The content of water-soluble content of crude drug shows that in pomegranate ethanolic extract there are more polar compounds than semi-polar and non-polar compounds, although the three classes of compounds are contained in pomegranate ethanol extract.

Table 2: Result of Characterization and Standardization of Crude Drug.

Parameter	Result (% w/w)
Water-soluble content	41.86 ± 0.06
Ethanol soluble content	37.18 ± 0.84
Water content*	5.33
Loss on drying	6.215 ± 0.06
Total ash content	3.44 ± 0.002
Total ash water-soluble content	3.36 ± 0.007
Total ash insoluble in acid	0.4625 ± 0.530

(*) %v/w.

An acute toxicity study was performed on zebrafish embryos exposed to the test solution for 96 hours. This test aims to determine the value of LC_{50} extract, which is the value that is able to cause as much as 50 percent of test animals experience

death. The motility of zebrafish embryo is indicated if it has one of the four parameters i.e. coagulation, non-formation, heart not beating and the embryo's tail from the yolk is not formed. Based on the results of the analysis with 95% confidence interval and 5% alpha, obtained the LC_{50} value of pomegranate skin ethanol extract of 196,037 ± 9.2 µg/mL (Figure 1).

Table 3: Predicted Results of Endotoxicity, Cardiotoxicity, Hepatotoxicity, Skin and Respiratory Sensitivity, and Reproduction.

Compound	Endotoxicity		Cardio-toxicity	Hepatotoxicity					Skin and Respiratory	Reproduction
	Estrogen	Androgen		Alkaline Phosphatase	Gamma Glutamine Transferase	Lactate Dehydrogenase	SGOT	SGPT		
S1	-	-	-	+	+	+	+	+	+	-
S2	-	-	-	-	-	+	+	+	+	-
S3	-	-	-	-	-	+	-	-	+	-
S4	-	-	-	-	+	+	-	+	+	-
S5	-	-	-	-	+	+	-	-	+	-
S6	-	-	-	+	+	-	+	+	+	-
S7	+	-	-	-	+	+	-	+	+	-
S8	+	-	-	-	+	-	-	-	+	-
S9	+	-	-	-	-	-	-	-	-	-
S10	-	-	-	-	+	-	-	-	+	-
S11	-	-	-	-	-	+	-	-	+	-
S12	+	-	-	-	+	+	-	-	+	-
S13	-	-	-	+	+	-	+	+	+	-

+ = positive, - = negative.

Organization for Economic Co-operation and Development (OECD) and European Chemicals Bureau (ECB) categorize the toxicity of pollutants against zebra fish into harmful (10 mg / L < LC 50 < 100 mg / L), toxic (1 mg/L < LC 50 < 10 mg / L), and highly toxic (LC 50 < 1mg / L). Based on these categories, pomegranate ethanol extract is included in the safe category.

Research conducted by Vidal (2003) states that the level of acute toxicity of pomegranate extract is relatively low, thus it is safe to use as an alternative treatment. In this study, the value of LD_{50} pomegranate extract is 731.1 mg/kg with the model used are male and female rats. A study performed by Zhang (2003) found a correlation between zebrafish toxicity and mice. Another study conducted by Fuentes (1985) suggested that pomegranate extracts are relatively safe where part of the toxic plant of the pomegranate is the root and stem bark due to its toxic alkaloids content.

Prediction toxicity using ADMET Predictor 7.1 is used to determine the degree of safety of each compound contained in the skin of pomegranate. ADMET's prediction is a challenging process because the drug will deal with thousands of proteins during the process. Therefore, the ADMET determination method is usually based only on relatively simple databases, such as lipophilicity, pKa, and functional groups involved in the compound (Young, 2009).

Furthermore, computational chemistry is able to predict the parameters of absorption, distribution, metabolism, elimination, and toxicity of a compound. The program works by predicting these parameters by considering the molecular structure and the physicochemical properties of the compound. The results of toxicity prediction are listed in Tables 3 and 4.

Table 4: Predicted Results of Carcinogenicity, Mutagenitas, Aquatic Toxicity, and Environmental Toxicity.

Compound	Carcinogenicity[1]		Mutagenicity[2]										Aquatic Toxicity[3]			Environmental Toxicity[4]
	Rat	Mice	Compound					Metabolite					A	B	C	
			97 + 1537	98	100	102 + wp2	1535	97 + 1537	98	100	102 + wp2	1535				
S1	391	385	-	+	-	-	-	-	-	-	-	-	887	1.3	0.8	0.07
S2	80	305	+	-	-	-	-	-	-	-	-	-	63	0.2	408	3.4
S3	124	621	+	+	-	-	-	+	-	-	-	-	110	11.7	1609	0.15
S4	104	173	-	-	-	-	-	-	-	-	-	-	445	2.7	33348	1.61
S5	131	161	-	-	-	+	-	+	-	-	+	-	2	0.2	43	1.26
S6	67	152	-	-	-	-	-	-	-	-	-	-	23	1.1	623	2.62
S7	186	249	+	-	-	-	-	+	-	-	-	-	11	0.1	198	3.26
S8	214	260	+	-	-	-	-	+	-	-	+	-	6	0.22	221	2.22
S9	214	192	-	-	-	-	-	-	-	-	-	-	215	1	25	2.55
S10	97	1128	-	-	-	+	-	+	-	-	+	-	83	186	235	2×10^{-3}
S11	132	347	+	-	-	+	-	+	-	-	+	-	484	3.2	584	0.54
S12	190	233	-	-	-	+	-	+	-	-	+	-	5	9.1	19	3.55
S13	24	121	-	-	-	-	-	-	-	-	-	-	2	2×10^{-3}	4	48.4

[1]TD (*Tumor Dose*) 50 mg/kg/day [2]Mutagenicity Ames test toward *strain Salmonella* [3]A = *Pimephales promelai* (LC_{50} ,mg/L), B = *Tetrahymena puriformis* (IGC_{50}, mmol/L, dan C = *Daphnia magna* (LC_{50}, mg/L) [4]Bio Concentration Factor (BCF).

CONCLUSION

Based on aquatic toxicity classification by Organization for Economic Co-operation and Development (OECD), pomegranate peel ethanol extract was classified as safe. Toxicity prediction showed that brevifolin was the safest substances among the other substances that contained in ethanol extract of pomegranate peel with a binding energy of −4,02 kcal/mol. Brevifolin (S9) is the compound with the lowest toxicity level *in silico*. In addition, there is no compound that potentially causes problems in the reproductive organs, heart, and androgen hormones.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest is associated with this work.

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