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The use of computational chemistry to predict toxicity of antioxidants food additives and its metabolites as a reference for food safety regulation

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ABSTRACT

Antioxidant food additives are used to prevent or slowing down the oxidation process in foods. European Food Safety Authority (EFSA) by EC257/2010 regulation set up a program for reevaluation approved antioxidant food additives. Safety of the food additive was one of its concerns. The aim of this research was to predict antioxidant food additives toxicity using in silico toxicity prediction as preliminary evaluation of safety antioxidant food additives evaluation. The in silico prediction was conducted for acute toxicity (LD_{50}), mutagenicity, carcinogenicity, reproduction toxicity, chronic toxicity (NOEL), Acceptable Daily Intake (ADI) value and toxicity of Metabolites. The applied softwares were Toxtree, TEST, Admet Predictor and OECD QSAR Toolbox. Among 42 antioxidant food additives as chemicals test, the prediction methods predict that 6 compounds as carcinogen (carnosic acid, citric acid, ethylene diamine tetra acetate, isopropyl citrate, octyl gallate, and stearyl citrate); 2 compounds as mutagen (ascorbyl palmitate and 2,4,5-trihydroxybutyrophenone); 8 compounds as reproduction toxic (4 hexyl resorcinol, alpha tocopherol, butylated hydroxy anisole, delta tocopherol, ethoxyquine, gamma tocopherol, tertiary butyl hydroquinone); and 1 compound as mutagen and reproduction toxic, that is norhydroguairetic acid. Acute toxicity prediction was conducted by LD_{50} prediction. The lowest LD_{50} value was ethoxyquine, 937.84 mg/kg and the highest LD_{50}value was dilauryl thiodipropionate, 13367.79 mg/kg. The comparison between LD_{50} prediction and LD_{50} experimental was using paired t-test method. It is concluded that there is no significantly difference between LD_{50} prediction and LD_{50} experimental. Chronic toxicity prediction was conducted by NOEL value prediction, and ADI value was calculated from NOEL value. The lowest ADI value was carnosic acid (0.38 mg/kg bw/day) and the highest ADI value are calcium ascorbic and calcium disodium ethylen diamine tetraacetate (1.35 mg/kg bw/day). The comparison between ADI prediction and ADI experimental was using paired t-test method concluded that there is no significant difference between ADI prediction and ADI experimental. Metabolitee prediction was conducted using two softwares that are Toxtree and Admet Predictor. The Metabolitee prediction showed change in prediction result. The in silico toxicity prediction method can be used as one supportive method to perform food additive safety evaluation by prediction of carcinogen, genotoxicity, reproduction toxicity, LD_{50} value and ADI value.

Keywords: Food Additives, Antioxidants, Toxicity, Prediction, In Silico

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INTRODUCTION

Food additives is a substance that is intentionally added to food to influence the form or nature. Food additive is not intended to be consumed directly and not as food raw materials. Food additive may have or do not have nutritional

value, which is intentionally added to food for a technological purpose in the process of production to distribution to affect the nature of the food, either directly or indirectly (Codex, 1995).

One type of food additives that is often used is antioxidant. Antioxidants are food additives food to prevent damage due to oxidation. Safety evaluation of food additives should be done comprehensively covers toxicokinetic test, acute toxicity, subchronic toxicity, chronic toxicity, genotoxicity and carcinogenic, reproductive and growth toxicity, and other toxicity studies such as immunotoxicity, allergenicity, neurotoxicity, irritation and toxicity of the organ target.

Evaluation of the use of food additives safety is also done for approved and used food additive.European Food Safety Authority (EFSA) through regulation EC 257/2010 has made a re-evaluation program of food additive. All food additive which have been approved and used in Europe will be re-evaluated, one of which is antioxidant. In general,antioxidants will be reevaluated until December 31, 2018 and for antioxidants, such as gallate, BHA, BHT, propionic acid, tocopherol will be made until December 31, 2015.

Food additive evaluation is done through toxicity tests that can be performed by the method of in vitro and in vivo. Implementation of toxicity tests with these two methods require a high cost, in addition to the time it takes a longerprocess and requires the use of experimental animals.To reduce these barriers, development of in silico methods will beimplemented to more efficient, faster, no animal testing and nolarge budget.

In silico methods that have been and continue to be developed is to predict the toxicity of a compound based on chemical structure, such as (Quantitative) Structure Activity Relationship (Q) SAR. In silico methods (Q) SAR is a computational chemistry techniques that predict the activity of chemical compounds based on the mathematical relationship between physicochemical properties of compounds and their biological activities including toxicity effects (Valerio Jr., 2009, Milan *et al*, 2009; Roncaglioni *et al*, 2013, Toporov, 2014).

QSAR Method is widely used in the development of new drugs, but it is also used by regulatory agencies as one of the methods in decision making related to the toxicity of a compound, such as the USA EPA (The Environmental Protection Agency), US Department of Health and Human Services ATSDR (Agency for Toxic Substances The and Disease Registry), USA FDA CDER (Center for Drug Evaluation and Research), the Canadian Regulatory agencies, ECVAM (European Centre for the Validation of The Alternative Methods), ICCVAM (The Interagency Coordinating Committee on the Validation of Alternative Methods) and OECD (The Organization for Economic Co-operation and Development).

Recently, many softwaresare available to predict toxicity using models of algorithms and different databases. With the difference in the use of algorthma and databases, it is possible that there is a difference in outcome prediction of the toxicity of the compound. Many researchers perform a validation of the prediction methods used and compare methods of prediction between existing software to assess the accuracy and robustness of each software (Valerio, 2009; Valerio *et al*, 2012). Combining several different prediction methods will yield a more accurate prediction methods and robust compared with the use of the prediction method (OECD, 2007).

This study was aimed to predict the toxicity of food additive antioxidant compounds as initial evaluation of antioxidant food additivesafety.

MATERIALS AND METHODS

Prediction of antioxidants toxicity include acute toxicity, chronic toxicity (NOEL value), carcinogenicity, mutagenicity, reproductive toxicity and the value of the Acceptable Daily Intake (ADI) ((Benigni and Bossa, 2011, Benigni et al, 2013, Prieto, 2013, Muster, 2008; Sutter, 2013, Cronin*et al* 2003, Cronin *et al* 2008)). The toxicity prediction using four softwares, namely ToxTree v2.6.0, TEST v4.1, ADMET Predictor v7.0.0004, and The OECD QSAR Toolbox v3.2.

Verification of prediction methods for prediction of carcinogenicity, mutagenicity and reproductive toxicity refers to the OECD validation instructions. For acute toxicity predictions will be compared with the value of existing research, as well as chronic toxicity prediction (NOEL) will be compared with the results of existing research in the form of ADI value.

After getting methods that have been verified, then predictions toxicity towards food additives Antioxidants as the test compound were carried out. There are 42 primary and secondary antioxidant compounds to be predicted.

The compounds were predicted by their carcinogenicity, mutagenicity and reproductive toxicity. The antioxidant metaboliteswere predicted by the method of Cytochrome P450. LD_{50} prediction values were compared with the LD_{50} value of existing research results statistically by paired t-test method followed by categorizing using toxicity Lu's Base Toxicology.Long term toxicity was predicted by value of NOEL. NOEL predictive values were calculated from the predicted value of the ADI. ADI prediction results were compared with the value of the existing ADI statistically by paired t-test method. All prediction was used statistical method (Cooper *et al*., 1979; OECD, 2007; Ryback*et al*, 2014).

Tabel 1. Summary of Prediction ofToxicity

No	Name	Toxicity Acute	Prediction ofCarcinogen	Prediction of Mutagen	Toxicity Reproduction	NOEL	ADI	Conclusion
1	4-hexyl resorcinol	Moderately Toxic	-	-	+	844	0.844	+
2	alpha tocopherol	Moderately Toxic	-	-	+	817	0.817	+
3	Ascorbic acid	Slightly Toxic	-	-	-	1070	1.07	-
4	Ascorbyl palmitate	Moderately Toxic	-	+	-	916	0.916	+
5	Ascorbyl stearate	Slightly Toxic	+	-	-	834	0.834	+
6	BHA-2iso	Moderately Toxic	+	-	+	772	0.772	+
7	BHA	Moderately Toxic	+	-	+	772	0.772	+
8	BHT	Moderately Toxic	-	-	-	519	0.519	-
9	Ca ascorbate	Moderately Toxic	-	-	-	1350	1.35	-
10	Ca disodium EDTA	Moderately Toxic	-	-	-	1350	1.35	-
11	Ca hydrogen sulfite	Moderately Toxic	-	-	-	1230	1.23	-
12	Carnosic acid	Moderately Toxic	+	-	-	380	0.38	+
13	Citric acid	Slightly Toxic	+	-	-	1670	1.67	+
14	Delta tocopherol	Moderately Toxic	-	-	+	798	0.798	+
15	Dilauryl thiodipropionate	Slightly Toxic	-	-	-	308	0.308	-
16	Disodium EDTA	Moderately Toxic	-	-	-	673	0.673	-
17	Dodecyl gallate	Moderately Toxic	-	-	-	922	0.922	-
18	EDTA	Moderately Toxic	+	-	-	1500	1.5	+
19	Erythorbic acid	Slightly Toxic	-	-	-	1030	1.03	-
20	Ethoxyquin	Moderately Toxic	-	-	+	729	0.729	+
21	Gamma tocopherol	Moderately Toxic	-	-	+	946	0.946	+
22	Isopropyl citrate	Moderately Toxic	+	-	-	1300	1.3	+
23	L Tartaric acid	Moderately Toxic	-	-	-	1030	1.03	-
24	NDGA	Moderately Toxic	-	+	+	677	0.677	+
25	Octyl gallate	Moderately Toxic	+	-	-	839	0.839	+
26	Potassium ascorbate	Moderately Toxic	-	-	-	1170	1.17	-
27	Potassium bisulfite	Moderately Toxic	-	-	-	1040	1.04	-
28	Potassium lactate	Moderately Toxic	-	-	-	2190	2.19	-
29	Potassium metabisulfite	Moderately Toxic	-	-	-	1010	1.01	-
30	Potassium sulfite	Moderately Toxic	-	-	-	923	0.923	-
31	Propyl gallate	Moderately Toxic	-	-	-	470	0.47	-
32	Sodium erythorbate	Moderately Toxic	-	-	-	1040	1.04	-
33	Sodium ascorbate	Moderately Toxic	-	-	-	1170	1.17	-
34	Sodium hydrogen sulfite	Moderately Toxic	-	-	-	700	0.7	-
35	Sodium lactate	Moderately Toxic	-	-	-	1110	1.11	-
36	Sodium metabisulfite	Moderately Toxic	-	-	-	1230	1.23	-
37	Sodium sulfite	Moderately Toxic	-	-	-	1160	1.16	-
38	Sodium thiosulphate	Moderately Toxic	-	-	-	1180	1.18	-
39	Stearyl citrate	Slightly Toxic	+	-	-	917	0.917	+
40	Tertiary butyl hydroquinone	Moderately Toxic	-	-	+	576	0.576	+
41	THBP	Moderately Toxic	-	+	-	747	0.747	+
42	Thiodipropionic acid	Slightly Toxic	-	-	-	984	0.984	-

+ Toxic - Non toxic

RESULTS AND DISCUSSION

Structure Activity Relationship (SAR) is a basic method in the in silico prediction of toxicity. The development of this method is marked with the number of database development and computer programs, but show different accuracy (Roncaglioni *et al*, 2013). Therefore,the verification is necessary. Prediction of Toxicity method is a

method that uses a classification model to determine a compound toxic or not. Verification method using classification models can be made by Cooper parameter Statistics.

Parameter Statistics Cooper can show the performance of a classification model by measuring its ability of a method to detect sensitivity,specificity and accuracy or concordance (OECD, 2007). In addition, the ability of a classification model can be determined from the value of Receiver Operating Characteristic (ROC) and robustness (Valeria *et al*, 2012; OECD, 2007). Prediction of toxicity results are summarized in Table 1. In this table, it is known that 17 antioxidant compounds that are toxic and the remaining 25 are not.

Prediction of metabolite antioxidants are divided into two groups for positive prediction and food negative predicted antioxidants. Seventeen postive predicted compound were analyzed their metabolic processes by cytochrom P450 using ADMET Predictor and Toxtree. Prediction of metebolite toxicity includes carcinogenicity, mutagenicity and reproduction toxicity. Results metabolite and its prediction of toxicity can be seen in Table 2.

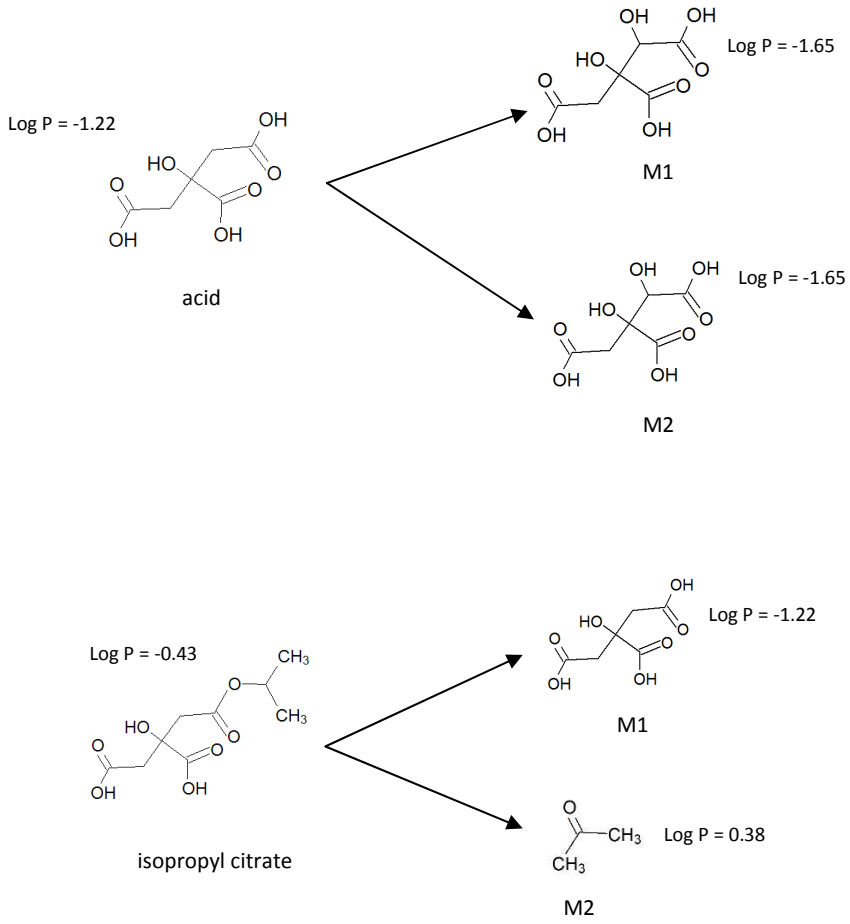
Tabel 2. Result of Metabolitesand Prediction ofToxicity of Positive Predicted Toxic Antioxidants

No	Name	Metabolite	Prediction ofCarcinogen	Prediction of Mutagen	Toxicity Reproduction	Conclusion
1	4-hexyl resorcinol	4HR-M1	+	+	+	+
		4HR-M2	+	+	+	+
2	alpha tocopherol	ATC - M1	-	-	+	+
		ATC - M2	-	-	+	+
		ATC - M3	-	-	+	+
		ATC - M4	-	-	-	-
		ATC - M5	-	-	+	+
3	Ascorbyl palmitate	M1	-	-	-	-
		M2	-	-	-	-
		M3	-	-	-	-
		M4	+	+	-	+
		M5	-	-	-	-
4	BHA-2iso	BHAiso-M1	-	-	+	+
		BHAiso-M2	+	+	+	+
		BHAiso-M3	+	+	+	+
5	BHA	BHA-M1	+	+	+	+
		BHA-M2	-	-	+	+
		BHA-M3	+	+	+	+
		BHA-M4	+	+	+	+
6	Carnosic acid	CNS-M1	-	-	-	-
		CNS-M2	-	-	-	-
7	Citric acid	M1	-	-	-	-
		M2	-	-	-	-
8	Delta tocopherol	M1	-	-	+	+
		M2	-	-	+	+
		M3	-	-	+	+
		M4	-	-	+	+
9	EDTA	M1	-	+	-	+
		M2	-	-	-	-
		M3	+	+	-	+
		M4	-	-	-	-
		M5	-	+	-	+
		M6	-	+	-	+
10	Ethoxyquin	M1	-	+	+	+
		M2	-	-	+	+
		M3	-	-	+	+
		M4	-	-	+	+
11	Gamma Tocopherol	M1	-	-	+	+
		M2	-	-	+	+
		M3	-	-	+	+
		M4	-	-	+	+
12	Isopropyl citrate	isoprop-M1	-	-	-	-
		Isoprop-M2	-	-	-	-
13	NDGA	NDGA-M1	+	+	-	+
		NDGA-M2	+	+	-	+
14	Octyl Gallate	M1	-	+	+	+
		M2	-	+	-	+
		M3	-	+	+	+

		M4	-	+	+	+
15	Stearyl citrate	M1	-	-	-	-
		M2	-	-	-	-
		M3	-	-	-	-
		M4	-	-	-	-
16	Tertiary Butyl Hydroquinone	M1	+	-	+	+
		M2	+	-	+	+
17	THBP	THBP-M1	-	+	-	+
		THBP-M2	-	+	-	+
		THBP-M3	-	+	-	+

+ Toxic - Non toxic

According to the results in Table 2, it can be concluded that in general the compound metabolite through cytocrom P450 metabolism is still a toxic compound, except carnosic acid, citric acid, isopropyl citrate and stearyl citrate.
Prediction of metabolic cytochrome P450, carnosic acid, citric acid, isopropyl citrate and stearyl citrate formed metabolite compounds that have different physicochemical values, as shown in Figure 1. The difference in the physicochemical properties of these will influence the calculation of the current descriptor.



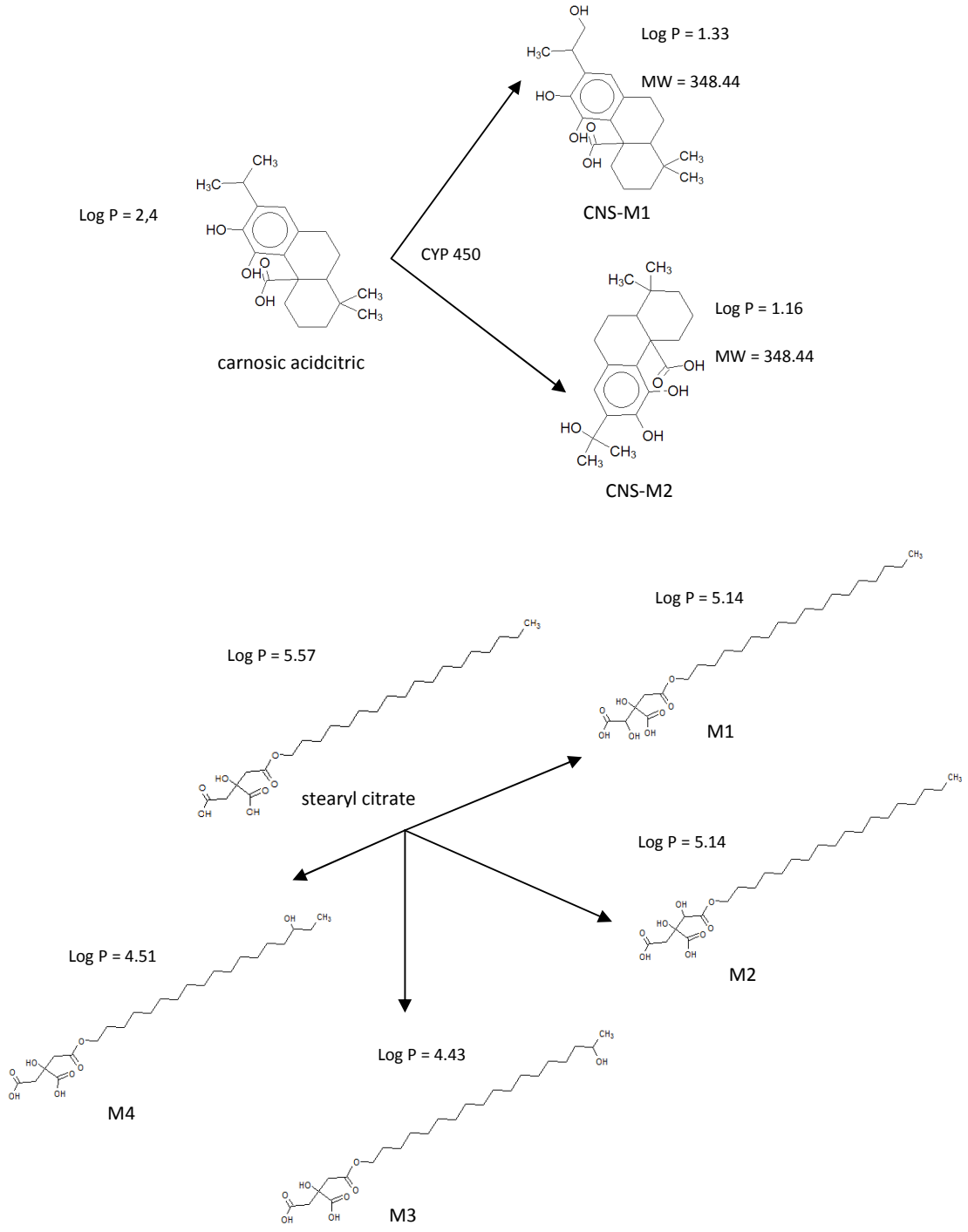


Figure 1. Deskriptor of Metabolites

Negative predicted antioxidants were further examined their metabolites toxicity. Results metabolite and its Prediction of Toxicity can be seen in Table 3.

From the results, it can be concluded that through cytocrom p450 metabolism, metabolite produced shows toxic and non-toxic compound. Interaction of xenobiotic compounds by metabolic enzymes will cause changes in biological effects, decrease or increase toxic activity (Guengerich, 2008).

Tabel 3. Result of Metabolitesand Prediction of Toxicity of Negative Predicted Toxic Antioxidants

No	Name	Metabolite	Prediction ofCarcinogen	Prediction of Mutagen	Toxicity Reproduction	Conclusion
1	Ascorbic acid	M1	+	+	-	+
		M2	-	-	-	-
		M2	-	-	-	-
2	Ascorbyl stearate	M1	+	-	-	+
		M2	+	-	-	+
		M3	+	-	-	+
		M4	+	-	-	+
3	BHT	M1	-	-	-	-
		M2	+	+	+	+
		M3	-	-	-	-
		M4	-	-	-	-
		M5	-	-	-	-
		M6	-	-	-	-
		M7	-	-	-	-
4	Dodecyl gallate	M1	-	+	+	+
		M2	-	+	+	+
		M3	-	+	-	+
		M4	-	+	+	+
5	Erythorbic acid	M1	+	+	-	+
		M2	-	-	-	-
		M3	-	+	-	+
6	L Tartaric acid	M1	-	-	-	-
		M2	-	-	-	-
7	Propyl gallate	M1	+	+	+	-
		M2	+	+	+	-
		M3	+	-	+	+
		M4	-	-	-	-
		M5	+	+	+	+
		M6	+	-	+	+
8	Thiodipropionic acid	M1	-	-	-	-
		M2	-	-	-	-
		M3	-	-	-	-
		M4	-	-	-	-
		M5	-	-	-	-

+ Toxic - Non toxic

Metabolic processes are complex reactions involving many enzymes and still its mechanism is little known. In general, metabolic process consists of two phases, namely phase I (functionalization reactions) and phase II (conjugation reaction). In the first phase of Xenobiotic compounds will undergo influx of new functional groups, the conversion of existing functional groups or decomposition through oxidation, reduction, and hydrolysis. Furthermore, in the second phase of compounds that have been metabolized in phase one will form conjugates with endogenous compounds body. Cytochrome enzyme (CYP) enzymes monooxigenase influence the process of phase I metabolism, approximately 75% of the total metabolism. (Guengerich, 2008)

ADMET Predictor software predict substrates for CYP isoforms 5 forms using ANNs of specific active atomic positions in metabolic reactions, while the software Toxtree SMARTCyp model uses DFT activation coupled with topological descriptors in determining soms CYP enzymes (CYP3A4 and 2D6) (Rydberg *et al*, 2010; Kirchmair *et al*, 2012). View of the complex metabolic processes, the results of ADMET Predictor Prediction and Toxtree only describe one of the stages of metabolism, so realibilitasnya therefore it is still limited to describe the process of metabolism.

CONCLUSION

1. In silico methods can be used to predict the toxicity of antioxidants, and the results showed that the predictive value of LD_{50} and ADI showed no significant differences with LD_{50} values and ADI literature.

2. 25 compounds of antioxidants are not toxic, and the remaining 17 compounds, predicted to have toxic properties, namely 6 compounds are carcinogenic, 2 mutagenic, 6 reproductive toxic, 2 carcinogen and reproduction toxic and 1 mutagenic and reproductive toxic.

3. The method used in silico may predict the phase I metabolism of compounds Food additives Antioxidants

4. The silico prediction of toxicity may be one method of support for security evaluation.

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