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Prediction of Carcinogenic, Mutagenic, Hepatotoxic, and LD_{50} Toxicity of Herbs *Euphorbia hirta* and *Camellia sinensis* Leaf Compounds as In Silico Antihypertensive Agents

Lusi Agus Setiani[1], Bina Lohita Sari[1*], Windry Muntaza[1]

[1] Pharmacy Department, Faculty of Mathematics and Natural Sciences, Pakuan University, Bogor, Indonesia.

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Corresponding Author:
Bina Lohita Sar
Binalohitasari@unpak.ac.id

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Abstract: Hypertension is defined as an increase in systolic blood pressure above 140 mmHg and diastolic blood pressure above 90 mmHg. Treatment for hypertension is lifelong, and continuous use of conventional medications can lead to the accumulation of drugs, potentially cautilizing adverse effects on the body compared to traditional treatments. The herbal tea formulation of *Euphorbia hirta* herbs and *Camellia sinensis* leaves can lower blood pressure due to their high antioxidant activity, which is associated with hypertension-related diseases, including compounds like quercetin and myricetin. This research serves as an initial screening to assess safety and minimize side effects of the active compounds in E. *hirta* and C. *sinensis*, which are beneficial as antihypertensive agents, through in silico analysis. Toxicity predictions utilizing the ProTox II, pkCSM, and Vega-QSAR platforms, which have been verified to meet the acceptability criteria of Cooper statistics parameters, were conducted. The toxicity assessment parameters include carcinogenicity, mutagenicity, hepatotoxicity, and acute toxicity (LD_{50}). The research results indicate that 9 compounds are carcinogenic and mutagenic, while 2 compounds are hepatotoxic. Based on the toxicity prediction assessment (LD_{50}), it was found that quercetin and myricetin, with moderate toxicity, have LD_{50} values of 159 mg/kgBW.

Keywords: Antihypertensive; *Camellia sinensis; Euphorbia hirta; In silico*; Toxicity.

Introduction

Hypertension is a degenerative disease characterized by an increase in systolic blood pressure of more than 140 mmHg and diastolic blood pressure of more than 90 mmHg, nicknamed "the silent disease" according to its sudden arrival and without showing any particular symptoms (Suiraoka, 2012). Hypertension disease is a global issue with a high prevalence estimated at 22% of the world's total population. Based on the results of the 2018 RISKESDAS survey, the prevalence of hypertension in Indonesia nationally stands at 34.11%, which is higher than the 2013 prevalence of 25.8% (Kemenkes RI, 2018). Hypertension treatment needs to be lifelong, and long-term conventional the consumption of narcotics has the potential to result in to the accumulation of continuous medication and potentially harmful side effects compared to traditional treatments, which tend to have relatively fewer side effects if used appropriately and rationally (Maesaroh *et al*, 2013). The World Health Organization (WHO) recommends the use of traditional medicine such as herbal medicine in maintaining public health to prevent and treat diseases, especially such as cancer, degenerative diseases, and chronic diseases (Setiawati *et al*, 2016).

Knowledge of medicinal plants in Indonesia has been passed down from one generation to the next. The

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experience and skills in traditional medicine have proven beneficial as alternative treatments. Based on the results of the 2018 RISKESDAS data, it shows that 59.12% of the Indonesian population still consumes herbal remedies, and 95.6% of herbal users acknowledge the benefits of herbal remedies for their health (Kemenkes RI, 2018). Herbal concoctions from plants such as *Curcuma xanthorrhiza, Orthosipon stamineus, Centella asiatica, and Soncus arvensis* are also used as hypertension treatments and have demonstrated effectiveness while being categorized as practically non-toxic (PNT) substances with no mortality and obtained LD50 > 50190mg/kgbw. Administration of this hypertensive herb at all doses for 45 days and 90 days, did not cause abnormalities in blood, liver and kidney functions (Winarno *et al*, 2015). Consuming herbal combinations in hypertensive patients reduces the risk of long-term mortality and morbidity so that hypertension treatment can last a lifetime (Steddon et al, 2014).

Herbal tea formulations from *Euphorbia hirta* and *Camellia sinensis* in Kalimantan are also widely used as an alternative treatment in lowering blood pressure. Bioflavonoid compounds, quercitrin, and myricitrin, found in these plants, have dual benefits as sources of antioxidants. (DJKI, 2021). Compounds with high antioxidant activity play a role and have a relationship with inflammation, oxidative stress, and endothelial dysfunction in hypertension-related diseases (Dinh *et al*, 2014). The use of herbal medicines is increasing so that scientific research is needed on efficacy, safety and quality standards so that their use is in accordance with established quality standards. Information related to the safety of these plants is still limited so more toxicity testing needs to be done to meet these quality standards. WHO also supports efforts to improve the safety and efficacy of traditional medicines (WHO, 2003). Using in silico computational methods as screening can provide preliminary information and identify the safety of a compound with a short time and relatively less cost compared to conventional methods.

The assessment of toxicity prediction uses algorithmic models, software, and data to analyze or predict the toxicity of a chemical compound (Raies and Bajic, 2016). Based on the description above, it is necessary to perform toxicity prediction related to the safety information of E.*hirta* and C.*sinensis* compounds as antihypertensive agents in silico utilizing online-based software, namely ProTox II, pkCSM, and the Vega-QSAR platform with parameters including carcinogenic, mutagenic, hepatotoxic, and acute toxicity (LD_{50}).

Method

This research employed hardware in the form of a Samsung Electronics laptop with the following specifications: AMD E-300 APU processor, Radeon (TM) HD Graphics 1.30 GHz, 4 GB RAM, and Windows 10 Pro 64-bit. Testing was conducted utilizing online-based software such as ProTox II (Prediction of Toxicology of Chemicals) accessible at (https://tox new.charite.de/protox_II/), pkCSM (Predicting Small-Molecule Pharmacokinetics and Toxicity Properties Utilizing Graph-Based Signatures) accessible(https://biosig.lab.uq.edu.au/pkcsm/predict ion), and the Vega-QSAR GUI 1.2.0 platform. Literature search in this research was conducted through Google Scholar utilizing keywords *Euphorbia hirta*, *Camellia sinensis*, antihypertensive, and in silico toxicity. Positive control compounds and negative control compounds were fulfiled from the 2008 Classification, Labeling, and Packaging (CLP) data for carcinogenic and mutagenic compounds, while for hepatotoxic compounds, it was based on DILI-rank scores from the Food and Drug Administration (FDA) website accessed through (https://www.fda.gov/).

The first stage of the software was verified utilizing a confusion matrix to measure the compatibility of the prediction method with the acceptability assessment of Cooper's statistical parameters calculated utilizing the formula in Table 1. Cooper's statistics can demonstrate the performance of a classification model by assessing the ability of a method to predict toxic compounds (sensitivity), non-toxic compounds (specificity), and all compounds in general (accuracy) (Valerio and Cross, 2012). Positive controls consisting of 20 compounds and negative controls consisting of 30 compounds were created based on the parameters of carcinogenic, mutagenic, and hepatotoxic toxicity.

The second stage of *E.hirta* and *C.sinensis* compounds were each predicted using verified protox II, pkCSM, and Vega-QSAR software. Prediction parameters include carcinogenic, mutagenic and hepatotoxic. Then, compounds that are carcinogenic, mutagenic and hepatotoxic are predicted for LD50 toxicity using Protox II software. The following is the flow of toxicity prediction in this study, which can be seen in Figure 1.

Table 1. Cooper's Statistics

Cooper's Statistical Parameters	Description
Sensitivity (True Positive Rate) TP/(TP+FN)	The proportion of toxic compounds correctly predicted,
Specificity (True Negative Rate) TN/(FP+TN)	The proportion of non-toxic compounds correctly predicted,
Accuracy (TP+TN)/(TP+FN+FP+TN)	The proportion of compounds correctly predicted by the toxicity prediction method.
Positive Predictivity TP/ (TP+FP)	The values of positive prediction results correctly indicating toxic compounds,
Negative Predictivity TN/(FN+TN)	The values of negative prediction results correctly indicating non-toxic compounds,
False Positive Rate FP/(FP+TN)	The proportion of non-toxic compounds predicted incorrectly as toxic compounds,
False Negatve Rate FN /TP+FN	The proportion of toxic compounds predicted incorrectly as non-toxic compounds,
Receiver Operating Characteristic TP/FP or TN/FN	The value of correct predictions (TP or TN) compared to incorrect predictions (FP or FN).

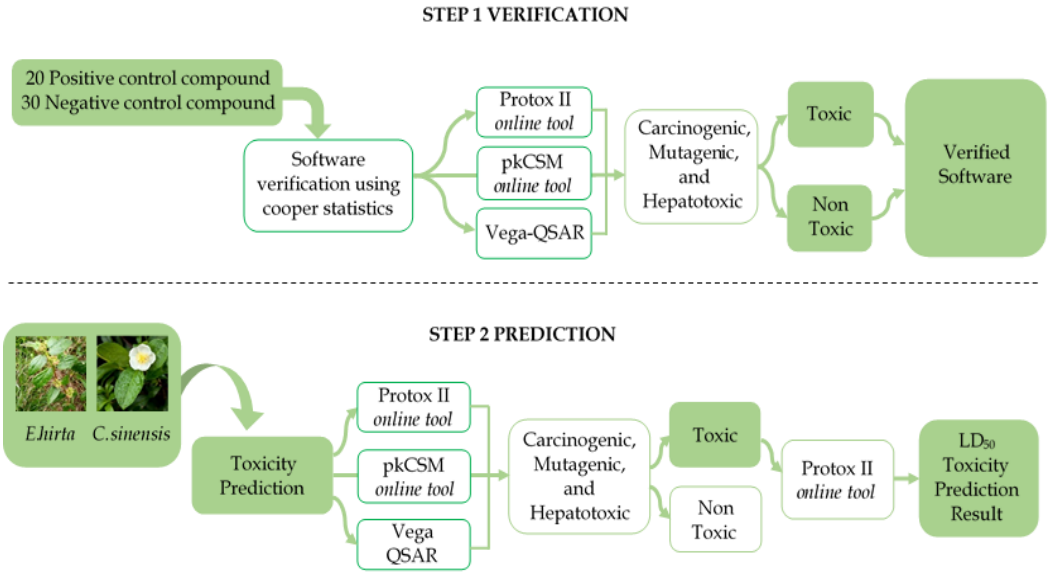


Figure 1. Research Flow

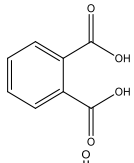
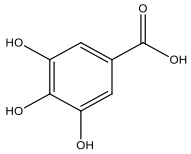
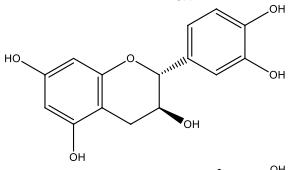
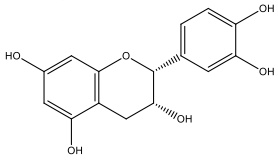
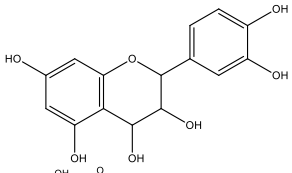
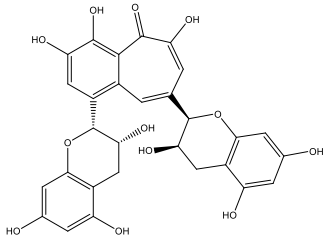
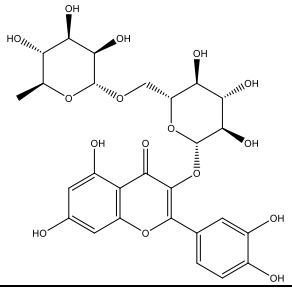
Result and Discussion

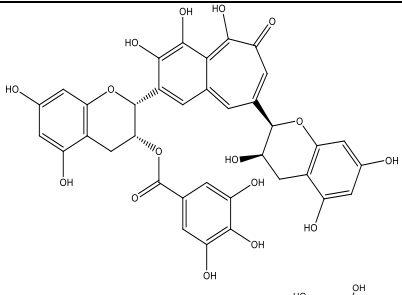
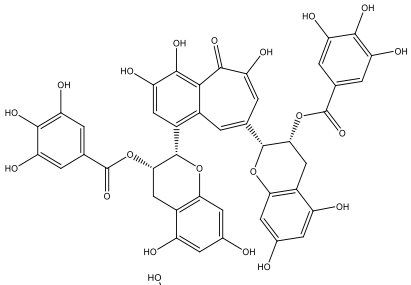
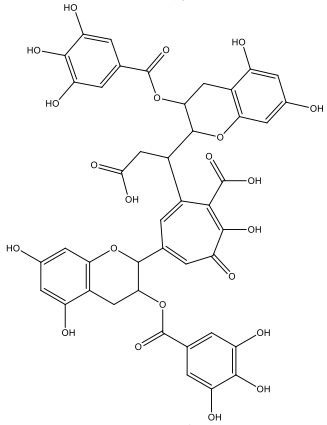
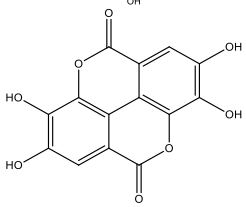
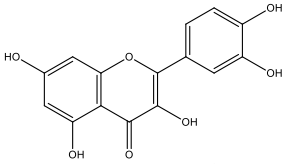
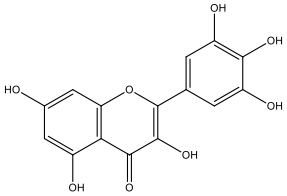
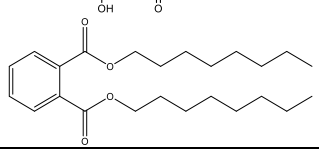
Toxicity predictions were verified because the interpretation methods of prediction results in each software are different. This verification method is suitable for prediction models based on classification to determine whether a compound is positive (toxic) or negative (non-toxic). Cooper's statistics are capable of demonstrating the performance of a classification model by assessing the ability of a predictive method for toxic compounds (sensitivity), non-toxic compounds (specificity), and all compounds in general (accuracy). (Valerio and Cross, 2012). The verification results indicate that the assessment based on Cooper's statistics in ProTox II is superior in prediction compared to pkCSM and Vega-QSAR. All evaluation aspects in these three software tools meet Cooper's statistics criteria based on the Organisation for Economic Co-operation and Development (OECD), which means values greater than >0.5 for sensitivity, specificity, accuracy, positive prediction, and negative prediction parameters. Meanwhile, the false positive rate and false negative rate are <0.5. Additionally, the prediction will demonstrate significant discriminative ability by providing an ROC value >2 (Valerio and Cross, 2012), there by making it suitable for predicting test compounds.

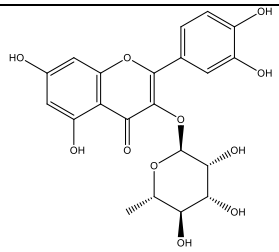
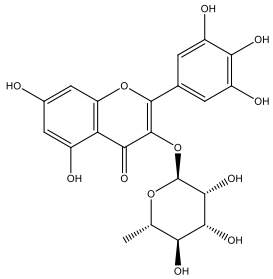
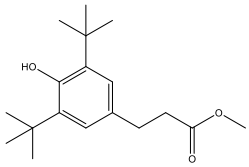
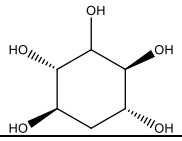
To assess the potential toxicity of a compound early on can enhance its safety. Therefore, it is necessary to conduct toxicity prediction for the compounds of *Euphorbia hirta* herbs and *Camellia sinensis* leaves, which are used as alternative treatments for hypertension. Toxicity prediction comprises tests for carcinogenicity,

mutagenicity, hepatotoxicity, and lethal dose 50 (LD_{50}). The test compounds E. *hirta* and C. *sinensis* used in this toxicity prediction were fulfiled from various studies, such as: (Setiani *et al*, 2022; Linfang *et al*, 2012; Masruroh & Tukiran, 2017; Ahmad, 2013; Khan & Mukhtar, 2013). The following is the result of toxicity data for compounds from the E. *hirta* and C. *sinensis*, which are carcinogenic, mutagenic, and hepatotoxic, as can be seen in Table 2.

Table 2. Data for Predicted Toxicity of Compounds

Compound Name	BM (g/mol)	Compound Structure	Toxicity Information
Phthalic Acid	166.13		Carcinogenic
Gallic Acid	170.12		Carcinogenic
Catechin	290.27		Carcinogenic
Epicatechin	290.27		Carcinogenic
Leucocyanidin	306.27		Carcinogenic
Theaflavin	564.50		Carcinogenic and mutagenic
Rutin	610.50		Carcinogenic and mutagenic

Theaflavin-3-gallate	716.60		Carcinogenic and mutagenic
Theaflavin-3-3-digallate	868.70		Carcinogenic and mutagenic
Thearubigin	902.70		Carcinogenic and Mutagenic
Ellagic Acid	302.19		Carcinogenic
Quercetin	302.24		Carcinogenic and Mutagenic
Myricetin	318.23		Carcinogenic and Mutagenic
di-n-octyl Phthalate	390.60		Carcinogenic

Quercitrin	448.40		Carcinogenic and Mutagenic
Myricitrin	464.40		Carcinogenic and mutagenic
Methyl-3-(3,5-ditertbutyl4-hydroxyphenyl) propionate	292.40		Hepatotoxic
Quercitol	164.16		Hepatotoxic

Based on the results shown in Table 3 above, 18 toxic compounds were identified. In the carcinogenicity testing, the ProTox II software identified 10 compounds, including quercetin, leucocyanidin, quercitrin, myricitrin, ellagic acid, gallic acid, di-n-octyl phthalate, catechin, epicatechin, and myricetin. Meanwhile, the Vega-QSAR software found 12 compounds, including quercetin, quercitrin, myricitrin, ellagic acid, kaempferol, di-n-octyl phthalate, theaflavin, theaflavin-3-gallate, theaflavin-3-3-digallate, thearubigin, myricetin, and phthalic acid. From the prediction results utilizing both software tools, 6 compounds were found to be carcinogenic, including quercetin, quercitrin, myricitrin, ellagic acid, di-n-octyl phthalate, and myricetin. In the mutagenicity testing, ProTox II and Vega-QSAR identified two compounds, namely quercetin and myricetin. Subsequently, prediction results utilizing Vega-QSAR revealed nine compounds, including quercetin, quercitrin, myricitrin, rutin, theaflavin, theaflavin-3-gallate,theaflavin-3-3-digallate, myricetin, and thearubigin. Meanwhile, the toxicity prediction utilizing pkCSM did not identify any mutagenic compounds. In the hepatotoxicity testing, the pkCSM online tool found one compound, namely quercitol, while the ProTox II online tool identified one compound, which is methyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate.

Toxicity and Molecular Weight

This research compares toxicity based on molecular weight with prediction results, including carcinogenic toxicity utilizing the ProTox II and Vega-QSAR prediction tools, mutagenic prediction utilizing ProTox II, pkCSM, and Vega-QSAR, and hepatotoxicity utilizing the ProTox II and pkCSM software. The following are the results of toxicity prediction comparisons supported by verified software in their respective toxicity categories, including carcinogenic, mutagenic, and hepatotoxic, as seen in Figure 2 below.

Toxicity prediction based on molecular weight in Figure 2 reveals that thearubigin compound has the highest molecular weight and falls into two toxicity categories, namely carcinogenic and mutagenic, whereas the quercitol compound is found to be hepatotoxic. This result aligns with the observed trend that hazardous substances exhibit greater molecular mass (Struck et al, 2008). The excretion of a compound is favorable when it has a low molecular weight and is hydrophilic. Conversely, if the molecular weight is high and it exhibits hydrophobic properties, the excreted compound becomes smaller, potentially cautilizing toxicity (Shofi, 2022).

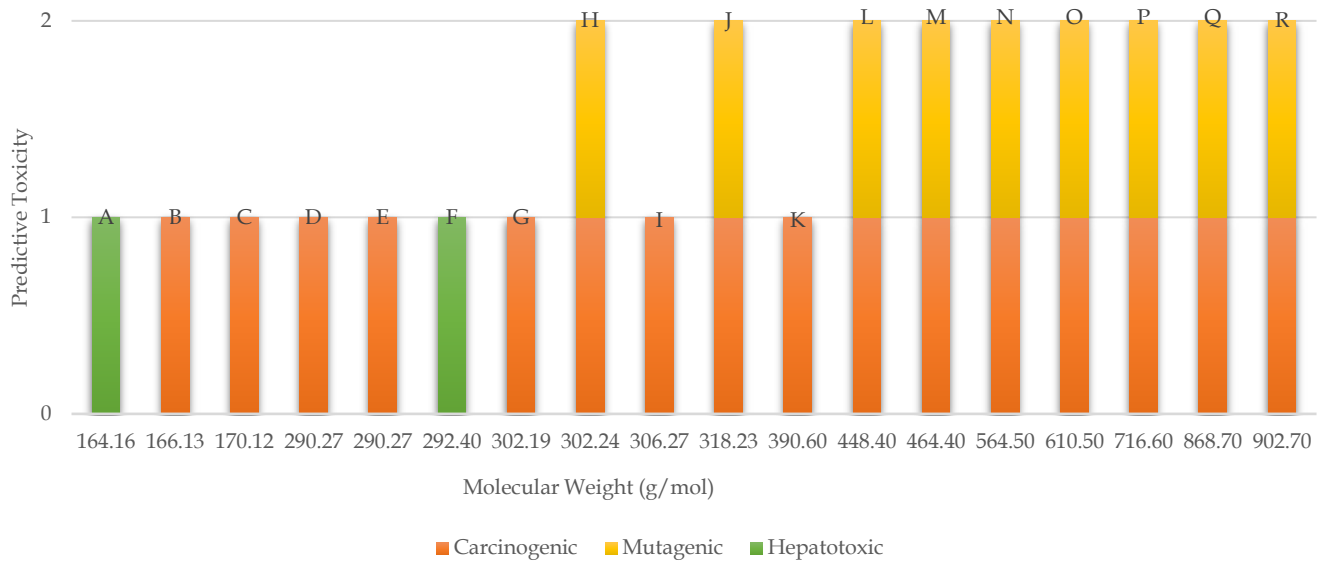


Figure 2. Toxicity and Molecular Weight

Note: 1 = one category of toxicity; 2 = two categories of toxicity

Description:

(A) Quercitol, (B) Phthalic Acid, (C) Gallic Acid, (D) Catechin, (E) Epicatechin, (F) Methyl-3-(3,5-ditertbutyl4-hydroxyphenyl) propionate, (G) Ellagic Acid, (H) Quercetin, (I) Leucocyanidin, (J) Myricetin, (K) di-n-octyl Phthalate, (L) Quercitrin, (M) Myricitrin, (N) Theaflavin, (O) Rutin, (P) Theaflavin-3-gallate, (Q) Theaflavin-3-3-digallate and (R) Thearubigin.

The toxicity prediction results utilizing the Vega-QSAR platform are implemented with rule-based prediction models created utilizing various sets of compounds, different molecular descriptors, and different algorithms. For each model, Vega provides predictions and measures the prediction reliability through the Application Domain Index (Benfentatia et al, 2023). Furthermore, in the development of the QSAR platform Vega-QSAR, it is based on the determination of structure alerts in compounds labeled as toxic or non-toxic (Bhat and Chatterjee, 2021). Structure Alert refers to molecular substructures or reactive groups associated with the carcinogenic and mutagenic properties of chemicals (Plošnik et al, 2016).

In the carcinogenicity testing utilizing the Vega-QSAR platform, structure alerts were found for compounds H, J, L, M, and O, including SA no. 48, which falls under the Quercetin-type flavonoids category. Several other compounds, such as compound H, exhibited Structure Alert no. 47, which is O-Phenyl phenol, Structure alert O-phenylphenol or Ortho-phenyl phenol (OPP) compounds are hazardous chemicals that can react with strong bases and strong oxidants that decompose on heating and produce carbon dioxide, carbon monoxide and irritating fumes (ILO and WHO, 2013). While compounds B and K had SA no. 42, corresponding to Phthalate diesters and monoesters. Lastly, SA no. 10, α, *β unsaturated carbonyls*, was detected in several compounds, including compounds M, N, and Q. Furthermore, in the mutagenicity testing, structure alert no. 60 Flavonoids was found in several compounds, including H, J, L, M, and O. Additionally, compounds N, P, Q, and R exhibited SA no. 10, α, *β unsaturated carbonyls*. The mechanism of carcinogen α, β unsaturated carbonyls is the same as the mutagenic mechanism, namely through oxidative stress. Oxidative stress is caused by an imbalance of Reactive Oxygen Species (ROS) and antioxidants in target cells. Reactive Oxygen Species (ROS) interact in cellular components such as lipids and DNA. Heavy metals such as As, Cu, Cr, Hg and Co are carcinogens in animals and humans (Ercal *et al*, 2001). And for hepatotoxicity testing utilizing ProTox II, the compound methy l-3-(3,5- di- tert- butyl- 4- hydroxy phenyl) propionate was found to be hepatotoxic, while the compound quercitol was identified in the pkCSM software.

In both software tools (ProTox II and pkCSM), structure alerts are not interpreted as an analysis model. The prediction model added to the ProTox-II platform is based on the Random Forest (RF) machine learning algorithm. The Random Forest (RF) model is assembled using 500 decision trees and employs the GINI index as the splitting criterion. An inherent advantage of employing the RF-based classifier is its proclivity for mitigating overfitting (Banerjee et al, 2018). Conversely, the utilization of pkCSM Graph-Based Signatures entails

the application of graph modeling, incorporating a well-established and rigorously validated mathematical representation of chemical entities. These versatile descriptors encompass various aspects of molecular structure, enabling the extraction of valuable chemical information. A straightforward graphical depiction of a chemical compound can be attained by representing individual atoms as nodes and their covalent linkages as edges in a graph. This elementary representation may be enhanced through the inclusion of annotations describing the physicochemical attributes of atoms and bonds. Additionally, it facilitates the investigation of structural patterns. Techniques for structural matching, such as toxicophore searching, subgraph mining, and graph analysis, can be effectively employed for this purpose (Pires et al, 2015).

Lethal Dose 50 (LD_{50})

Acute toxicity parameters with LD_{50} values are commonly used to predict toxicity. The LD_{50}, or Median Lethal Dose, represents the quantity of a substance at which 50% of a given population of test subjects succumb following exposure to the compound. The lethal dose (LD_{50}) classification is divided into six toxicity classes with threshold values of Highly Toxic ≤ 1 mg/kg, Toxic 1-50 mg/kg, Moderately Toxic 50-500 mg/kg, Mildly Toxic 500-5000 mg/kg, Practically Non-Toxic 5-15 g/kg, Relatively Harmless ≥15 g/kg body weight (BPOM RI, 2014). The prediction of acute toxicity utilizing ProTox II is a web server for predicting the oral toxicity of small molecules in rodents with the toxic dose LD_{50} in mg/kg body weight. The predictive outcomes are presented in Table 3 below, 11 compounds were identified from positive carcinogenic, mutagenic and hepatotoxic compound predictions.

Table 3. LD_{50} Prediction Results

Compound Name	Molecular weight (g/mol)	Lethal dose mg/kg	Class	Classification
Quercetin	302.24	159	3	Moderately Toxic
Quercitrin	448.40	5000	5	Practically Non-Toxic
Myricitrin	464.40	5000	5	Practically Non-Toxic
Rutin	610.50	5000	5	Practically Non-Toxic
Theaflavin	564.50	2500	5	Practically Non-Toxic
Theaflavin-3-gallate	716.60	1000	4	Mildly Toxic
Theaflavin-3-3-digallate	868.70	1000	4	Mildly Toxic
Thearubigin	902.70	1000	4	Mildly Toxic
Myricetin	318.23	159	3	Moderately Toxic
Quercitol	164.16	10000	6	Relatively Harmless
Methyl-3-(3,5-ditertbutyl4-hydroxyphenyl) propionate	292.40	5000	5	Practically Non-Toxic

The results of acute toxicity prediction (LD_{50}) for quercetin and myricetin classify them into the moderate toxicity category, specifically in toxicity class 3, with an LD_{50} value of 159 mg/kgbw. Several animal toxicity studies that support this classification indicate that quercetin can be tolerated at oral doses exceeding the oral LD_{50} value of 160 mg/kg body weight by several multiples (Chen et al, 2014). Furthermore, it is noteworthy that no adverse toxicity symptoms were documented in rabbits subjected to single intravenous administrations of quercetin, administered at doses ranging from 100 to 150 mg/kg of body weight, or through two injections, each not exceeding 136 mg/kg of body weight (Harwood et al, 2007). Meanwhile, a toxicity research involving intraperitoneal administration of myricetin at a dose of 1000 mg/kg body weight in rats did not indicate any toxic effects or mortality. This compound does not induce toxicity at doses above 100 mg/kg (LD_{50} value) in zebrafish larvae induced by ROS generated by UVB radiation. Very few of these studies raise concerns regarding side effects (Semwal et al, 2016). This suggests that LD_{50} toxicity values are expressed as estimates.

The compounds quercetin and myricetin in the carcinogenic testing exhibit structure alert no. 48, which is characteristic of Quercetin-type flavonoids. This structural alert is associated with flavonoids like quercetin, known for their antioxidant activity due to the presence of flavonoid groups. During the manifestation of its antioxidant properties, Quercetin has the potential to undergo transformation into oxidative byproducts, thereby posing a risk of oxidative degradation. This process may lead to the generation of an ortho-quinone and subsequent production of Reactive Oxygen Species (ROS), including superoxide and hydrogen peroxide (Boots et al, 2003). In the mutagenic testing, structure alert no. 60 Flavonoids was found in both compounds (quercetin and myricetin). This structural alert is more

closely associated with Quercetin-type compounds. The essential structural features relevant to the mutagenic activity of quercetin comprise the presence of a flavonoid ring structure bearing an unbound hydroxyl group at position 3, a double bond linking positions 2 and 3, and a ketone group situated at position 4. This structural arrangement enables the tautomerization of the hydroxyl group at position 3 into a 3-ketone moiety (Resende et al, 2012). However, in this research, the compounds quercetin and myricetin were not found to be hepatotoxic. Therefore, further experiments are needed to assess the toxicity of these compounds in order to enhance their safety.

Conclusion

The ProTox II software, pkCSM, and the Vega-QSAR platform have been verified to meet cooperative statistical parameter requirements and can be used as methods for predicting toxicity. The prediction results for the active compounds of *Euphorbia hirta* and *Camellia sinensis*, which are relatively low in carcinogenic and mutagenic properties but used as anti-hypertensive agents, include compounds such as quercetin and myricetin, which exhibit moderate toxicity with an LD_{50} value of 159 mg/kgBW at toxicity class level 3. And for compounds that are hepatotoxic, Methyl-3(3,5-di-tert-butyl-4-hydroxyphenyl) propionate can be hazardous if ingested, with an LD_{50} value of 5000 mg/kgBW and a toxicity class level of 5, while Quercitol, with a toxicity class level of 6, is non-toxic with an LD_{50} value of 10,000 mg/kgBW.

Authors Contribution

The first and second authors, Lusi Agus Setiani and Bina Lohita Sari contributed to developing the idea, exploring the data, analysing the data, guiding the research and writing the article. The third author, Windry Muntaza contributed to conducting research, exploring data, analysing data and writing this article. All authors have approved the final published manuscript.

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Conflicts of Interests

All authors declare no conflict of interest in this manuscript.

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