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in-silico PASS prediction for medicinal potency of antioxidant from *Ranunculus japonicus* Thunb.

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Abstract

Ranunculus japonicus has been traditionally used to treat various ailments, including malaria, jaundice, gastric problems, toothache, and eye inflammation. Recent studies reported that the plant extract effectively treat rheumatoid arthritis due to its secondary metabolites with antioxidant activities, such as flavonoids and phenols. In this study, we analyzed the flavonoids and phenols from *R. japonicus* using in silico methods. The study determined the flavonoid and phenol contents based on existing literature. The antioxidant potential analysis was conducted using PASS (*Prediction of Activity Spectra for Substances*). The results indicate the presence of two flavonoid compounds, namely Apigenin 6,8-di-C-|A-L-Arabinopyranoside and 3-Methylquercetin, and three phenol compounds, namely 3-Hydroxytrisol 3-O-glucoside, 4-Hydroxy-3-methoxyacetate, and Hydroxytrisol. Two compounds exhibited high bioactive potential (Pa value > 0.7), two compounds showed medium bioactive potential (Pa *value* > 0.5), and one compound exhibited low bioactive potential (Pa *value* < 0.5). The most bioactive compounds were Apigenin 6,8-di-C-|A-L-Arabinopyranoside and 3-Methylquercetin. The compounds with medium bioactivity were 3-Hydroxytrysol 3-O-glucoside and 4-Hydroxy-3-methoxycinnamic. The PASS online analysis revealed the potential of antioxidants for medicinal potencies.

Keywords: antioxidant, flavonoids, PASS-Online, phenols, *Ranunculus japonicus*

Introduction

Ranunculus japonicus is a perennial herbaceous plant belonging to the tribe Ranunculeae, family Ranunculaceae, and genus Ranunculus. The genus Ranunculus comprises 600 species (Yun et al., 2021; Azam et al., 2019). *R. japonicus* is distributed in East Asia and has various medicinal and culinary uses. In China, it is used to treat malaria, jaundice, gastric problems, toothache, and eye inflammation, while in Korea, the young stems are utilized as a vegetable (Yun et al., 2021; Zeng et al., 2021).

According to the Editorial Board of Flora of China (1978), *R. japonicus* thrives in mountainous environments and moist soil. Wang et al. (2021) have demonstrated that *R. japonicus* has the ability to treat rheumatoid arthritis due to its secondary metabolites, such as flavonoids and phenols. Flavonoids and phenols are well-known for their antioxidant activity (Tungmunnithum et al., 2018), which counteract the effects of free radicals (Pham-Huy et al., 2008). Free radicals are reactive compounds that can damage cells. The body naturally produces free radicals, such as reactive oxygen species (ROS) (Valko et al., 2007). However, when ROS production increases, it can cause damage to lipids, proteins, and DNA, leading to oxidative stress (Schieber & Chandel, 2014). Antioxidants scavenge various types of ROS, preventing or repairing damage caused by ROS (Valko et al., 2007).

The antioxidant activity of flavonoids and phenols has been widely demonstrated in various plants. Studies have shown that the extracted flavonoid content from *Petroselium crispum* leaves (Habtamu & Melaku, 2018) and Vernonia amygdalina flowers (Liberal et al., 2020) possess antioxidant activity. Similarly, Shim et al. (2020) found that Pyracantha angustifolia contains phenol content with antioxidant activity. However, it is still necessary to investigate the antioxidant activity of flavonoids and phenols present in R. japonicus.

Currently, secondary metabolite analysis can be conducted in silico. Databases containing secondary metabolite compounds in *R. japonicus* are easily accessible and can be used as references in the development of bioactive potential. This article utilizes an in-silico method to predict the potential of secondary metabolites using websites such as Pubchem and Way2drug. The Prediction of Activity Spectra for Substances (PASS) online is then used to determine the activity of these metabolites. The study aims to provide information

on the potential antioxidant properties of secondary metabolite compounds found in *R. japonicus*.

Materials and Methods

The study aimed to investigate secondary metabolite compounds from *Ranunculus japonicus* using in-silico methods. Antioxidant potential was determined based on a literature review. Chemical structures of compounds with potential antioxidant, antineoplastic, anticarcinogenic, and antiviral properties were obtained using SMILES (Simplified Molecular Input Line Entry System) from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/). The Prediction of Activity Spectra for Substances (PASS) (http://www.way2drug.com/PASSOnline/index.php) was used to perform in-silico analysis of the secondary metabolite compounds. This analysis aimed to determine Structure-Activity Relationships (SARs) and predict biological activities (Saharani et al., 2021). The resulting data included Pa (Potential activity) and Pi (potential inactivity) values, with the criteria of Pa > Pi ranging from 0.000 to 1.000 (Tomar et al., 2023). A Pa value greater than 0.7 indicates high biological activity of the compound, while a value between 0.5 and 0.7 indicates medium bioactivity. A value less than 0.5 indicates relatively low bioactivity

Results and Discussion

Based on the literature study, five secondary metabolite compounds were identified and classified into the flavonoid group and phenol group (Wang et al., 2021). The name, characteristics, and antioxidant potential are provided in Table 1.

Table 1. Metabolites and Pa values of *R. japonicus*

Secondary Metabolites	Compound	Molecular Weight (g/mol)	Organ	Antioxidant Potential		Antioxidant Activity	References
				Pa	Pi		
Flavonoids	Apigenin 6,8-di-C-|A-L-Arabinopyranoside	534.5	Leaf	0,851	0,003	Free radical scavenging	(Liberal *et al.*, 2020).
(Flavonols)	3-Methylquercetin	316.26	Leaf	0,809	0,003	Free radical scavenging	(Habtamu & Melaku, 2018)
Fenol	3-Hydroxytrysol 3-O-glucoside	316.30	Leaf	0,690	0,004	Anticancer	Chen *et al*, 2015
	4-Hydroxy-3-methoxycinnamic	194.18	Leaf	0,540	0,005	Anticancer	(Stompor-Goracy dan Machaczka, 2021).
	Hydroxytyrosol	154.16	Leaf	0,411	0,011	Anticancer	(Hadrich *et al.*, 2022).

Flavonoids

Flavonoids are a group of secondary metabolites primarily synthesized by plants (Dias et al., 2021). They have a basic flavonoid 15-carbon skeleton, C6-C3-C6, with two benzene rings (A and B) connected by a three-carbon pyran ring (C) (D'Amelia et al., 2018). Over 5000 flavonoids have been identified and they are classified into 10 categories based on their chemical structure. Six categories of flavonoids, including flavones, flavanones, anthocyanidins, flavonols, isoflavones, and catechins, are commonly found in human diets. Many of these flavonoids possess antioxidant activity, such as flavones and flavonols (Kocic et al., 2013). *R. japonicus* contains flavonoids such as Apigenin 6,8-di-C-|A-L-Arabinopyranoside and flavonols such as 3-Methylquercetin (Wang et al., 2021).

Apigenin 6,8-di-C-|A-L-Arabinopyranoside

The antioxidant potential of Apigenin 6,8-di-C-|A-L-Arabinopyranoside showed a Pa value of 0.851, indicating that it is a bioactive compound with a high likelihood of success when tested in vitro and/or in vivo (Malikhana et al., 2021). Apigenin 6,8-di-C-|A-L-Arabinopyranoside, extracted from *Petroselium crispum* leaves, has been proven to have high antioxidant activity. In vitro tests were performed using two cell-based assays: the oxidative haemolysis inhibition (OxHLIA) and thiobarbituric acid reactive substances (TBARS) assays. The results of the OxHLIA test indicated an IC50 value of 118 µg/mL for antioxidant activity at Δt = 60 minutes. Meanwhile, the TBARS assay showed antioxidant activity with EC50 values ranging from 1.5 to 1.6 mg/mL (Liberal et al., 2020).

Apigenin 6,8-di-C-|A-L-Arabinopyranoside is a natural compound of the C-glycosylflavone class (Wang et al., 2021). It is found in the leaves, specifically in the mesophyll tissue (Agati et al., 2012). This compound has been shown to possess antioxidant activity due to its free radical scavenging activity (ROS) and protective effects against oxidative stress both in vitro and in vivo (Han et al., 2012).

Reactive Oxygen Species (ROS) are molecules produced as byproducts of normal aerobic cellular metabolism in both plants and animals (Das et al., 2014). However, ROS are toxic and their accumulation can cause cellular macromolecular damage, such as protein and lipid oxidation, inhibition of cell proliferation, telomere DNA damage, and telomere shortening (Von et al., 2000). ROS accumulation is primarily caused by external factors such as pollution, smoke, drugs, and exposure to UV radiation (Juan et al., 2021). The ROS family consists mainly of hydrogen peroxide (H_2O_2), superoxide anion (O^{-}_2), and hydroxyl radical (OH^{-}) (McCance et al., 2006).

Apigenin 6,8-di-C-|A-L-Arabinopyranoside's free radical scavenging activity is associated with its hydroxyl groups at positions 4, 5, and 7. The antioxidant activity of apigenin can be attributed to the double bond between carbon atoms 2 and 3 in the C ring. Furthermore, the 4- and 5-hydroxyl groups in the A and C rings contribute to a higher level of radical scavenging capacity (He et al., 2019). Azimi et al. (2021) demonstrated that administering Apigenin 6,8-di-C-|A-L-Arabinopyranoside to rats with kidney stones protects tubular structures and inhibits intratubular crystal deposition induced by ethylene glycol and ammonium chloride treatment. The strong protective effect of Apigenin 6,8-di-C-|A-L-Arabinopyranoside on the enzymatic antioxidant system is due to its ability to scavenge free radicals, thus balancing antioxidants and ROS within the cells and enhancing endogenous antioxidant defence. Additionally, Apigenin intervention can increase the levels of glutathione reductase and the antioxidant enzyme superoxide dismutase (SOD) (Valdameri et al., 2011).

Buwa et al. (2016) demonstrated that Apigenin 6,8-di-C-|A-L-Arabinopyranoside can alleviate hemodynamic, biochemical, and histopathological changes in a rat model. The regulation of the antioxidant defence system and the enhancement of peroxisome proliferator-activated receptor gamma (PPARγ) signalling are involved in the mechanism. This regulates inflammatory responses and may represent potential therapeutic targets for preventing cardiac hypertrophy and metabolic disorders (Grande et al., 2021). Apigenin's activity can prevent lipid peroxidation caused by isopropanol, DNA damage, and antioxidant depletion in H9c2 cardiomyocytes (Thangaiyan et al., 2018).

3-Methylquercetin

The analysis of the antioxidant potential of 3-methylquercetin showed a Pa value of 0.809. The given value indicates that it is greater than 0.7, which makes it a bioactive compound with a high likelihood of success when tested in vitro and/or in vivo. To determine its antioxidant activity, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was conducted on 3-methylquercetin extracted from Vernonia amygdalina flowers, and the absorbance at 517 nm was measured. The test results indicate that 3-methylquercetin can inhibit radicals by 91.46% at 100 µg/mL, as demonstrated by the color change to yellow (Habtamu & Melaku, 2018).

3-Methylquercetin is a flavonol from the monomethoxyflavone group, which replaces the hydroxyl group at position 3 of quercetin with a methoxy group. According to Karancsi et al. (2022), this compound activates the Nrf2 (NF-E2-related factor 2) gene to enhance the binding of the antioxidant response element in the gene promoter region, resulting in antioxidant activity. Nrf2 regulates the antioxidant response element, which induces the expression of genes encoding

antioxidant proteins and phase II detoxification enzymes (Jeyapaul and Jaiswal, 2000). This leads to the production of various antioxidant proteins, including glutamate cysteine ligase and Sestrin2, which have a cytoprotective effect (Hye et al., 2014). In addition, isorhamnetin induces heme oxygenase-1, which reduces free radicals, leading to the inhibition of cyclooxygenase-2 and the inflammatory response (Seo et al., 2014).

The presence of 3-Methylquercetin to HCT116 cells exposed to CoCl2-induced oxidative stress can also reduce the production of reactive oxygen species (ROS), as measured by the intensity of DCFH-DA (2′,7′-dichlorofluorescin diacetate) fluorescence. The ROS measurement value in cells treated with 3-Methylquercetin is ±1.25, which is lower than that of the treatment group (±2.0). This also leads to the inhibition of HIF-1α (hypoxia-inducible factor-1) accumulation (Seo et al., 2016). HIF-1α is a key protein that regulates the growth and metastasis of H_2O_2-induced cancer cells (Semenza, 2003).

Phenol

Phenols are secondary metabolites produced by plants. They are involved in the adaptation processes of plants during stressful conditions such as injury, infection, or exposure to UV radiation (Vibhakar et al., 2021). Plants mainly produce phenols for growth, development, protection, and various physiological activities (Pratyusha, 2022). Phenolic compounds and plant extracts have demonstrated inhibitory effects on lipid oxidation caused by oxidative stress (Maqsood et al., 2014). *R. japonicus* contains several phenolic compounds, such as 3-Hydroxytrysol 3-O-glucoside, 4-Hydroxy-3-methoxycinnamic acid, and Hydroxytyrosol (Wang et al., 2021).

3-Hydroxytrysol 3-O-glucoside

The compound 3-Hydroxytrysol 3-O-glucoside, also known as cimidahurinine or CH, exhibits high antioxidant activity (Pa value of 0.690, Pubchem, 2023). This activity provides protection against doxorubicin (DOX)-induced cardiotoxicity (Chen et al., 2015) and prevents apoptosis mediated by oxidative stress by protecting membrane permeability. In a study conducted by Chen et al. (2015), treatment with cimidahurinine significantly reduced the percentage of apoptotic cells.

Shim et al. (2020) evaluated the therapeutic phytochemical bioactivity of Pyracantha angustifolia, which can reduce oxidation. Based on this research, cimidahurinine was found to have free radical scavenging effects and protein tyrosinase (TRY) activity. Cimidahurinine inhibited the formation of reactive oxygen species (ROS) in B16F10 cells and suppressed the expression of TRYP-1 and TRYP-2 proteins. TRYP-1 is a melanocyte-specific gene involved in melanin synthesis, while TRYP-2 acts as L-DOPAchrome to produce DHIICA through eumelanin synthesis. The inhibitory effect of cimidahurinine on the expression of TRYP-1 and TRYP-2 proteins was analyzed using a western blot. A Western blot analysis was performed to investigate the correlation between the inhibition of melanin production and antioxidant effects. The findings indicate that cimidahurinine effectively suppressed the formation of ROS in B16F10 cells caused by oxidation.

4-Hydroxy-3-methoxycinnamic

Ferulic acid, also known as 4-Hydroxy-3-methoxycinnamic acid or HMCA, is a polyphenol derivative of hydroxycinnamic acid. It is commonly found in rice, vegetables, fruits, and legumes and exhibits various antioxidant mechanisms, such as inhibiting reactive oxygen species, scavenging free radicals, and inhibiting lipid peroxidation (Ohue-Kitano et al., 2019; Chowdhury et al., 2016). 4-Hydroxy-3-methoxycinnamic acid is a compound found in leaves (Stompor-Goracy and Machaczka, 2021; Wang, Zhao-Yi., 2021). According to the PASS analysis, it has a Pa value of 0.540 and acts as a positive antioxidant compound to prevent damage caused by ultraviolet (UV) radiation and skin carcinogenesis (Stompor-Goracy and Machaczka, 2021).

Its antioxidant activity plays a role in reducing the harmful effects caused by lead acetate. Lead acetate is a common ingredient in cosmetics, hair dyes, and plant protective chemicals. Xu et al. (2021) conducted research that showed hydroxy-3-methoxycinnamic acid can protect AML-12 hepatocytes from palmitate-induced lipotoxicity by reducing ROS accumulation. This reduction in ROS accumulation can lower the activation of proinflammatory cytokines such as IL-6 and IL-1β. Liu et al. (2019) conducted an in vivo study on rats using TAC (total antioxidant capacity assay). The

study showed that hydroxy-3-methoxycinnamic acid has a positive effect on gut microbiota and improves heart function. Additionally, it restores the disrupted redox balance in pancreatic oxidative injury induced by iron (Stompor-Goracy and Machaczka, 2021).

Hydroxytyrosol (HT)

Hydroxytyrosol (HT) is a natural phenolic compound derived from the leaves of *R. japonicus* and Olea europaea (Hadrich et al., 2022; Pazos et al., 2008). It exhibits antioxidant activity and has been found to have efficient biological properties in combating obesity, cancer, diabetes, and inflammation (Bertelli et al., 2019; Hadrich et al., 2022).

HT compound induces the phosphorylation of Adenosine monophosphate-activated protein kinase (AMPK) in endothelial cells and activates the transcription factor FOXO3 in the nucleus, resulting in the expression of target genes such as catalase and Mn-superoxide dismutase. This expression is used to resist oxidative stress (Tejada et al., 2017). The activation of catalase expression is dependent on AMPK activation. Zrelli et al. (2011) found that HT compound induces AMPK phosphorylation, positively regulating the antioxidant defence system in endothelial cells.

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia, often associated with insulin resistance and beta cell dysfunction in the pancreas (Bellou et al., 2018; Petersmann et al., 2019). AMPK is an enzyme that plays a significant role in insulin resistance within T2DM. AMPK is an enzyme that regulates specific genes to reduce gluconeogenesis. According to Chellappan et al. (2018), AMPK activity in muscles improves glucose uptake, mitochondrial genes, and lipid oxidation.

Conclusion

R. japonicus presented five secondary metabolite compounds with antioxidant activities, i.e. Apigenin 6,8-di-C-|A-L-Arabinopyranoside, 3-Methylquercetin, 3-Hydroxytrysol 3-O-glucoside, 4-Hydroxy-3-methoxycinnamic acid, and Hydroxytyrosol. The antioxidant potential based on PASS online identified two compounds with a Pa value >0.7, two compounds with a Pa value >0.5, and one compound with a Pa value <0.5. The compounds with a higher biological activity were Apigenin 6,8-di-C-|A-L-Arabinopyranoside and 3-Methylquercetin. The compounds with a medium biological activity were 3-Hydroxytrysol 3-O-glucoside and 4-Hydroxy-3-methoxycinnamic acid, while the compound with the lowest biological activity was Hydroxytyrosol.

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