



Antioxidant Potential of Ethyl Acetate Fractionate from *Rhynchostylis retusa* (L.) Blume

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Abstract: - The escalation prevalence of degenerative diseases is closely related to excessive exposure toward free radicals, can bring about oxidative stress. The roots of the orchid tail may contain novel bioactive sources that act as radical scavengers. The purpose of this study was to find out the antioxidant potential of foxtail orchid roots (*Rhynchostylis retusa* (L.) Blume). The method utilized to identify new antioxidant potential using bioassay-guided fractionation. The root simplicial powder was extracted with methanol and then partitioned into *n*-hexane, ethyl acetate, dichloromethane, and water. The antioxidant activity exhibited that the ethyl acetate extract had IC₅₀ value 33.47 µg/mL compared to the *n*-hexane, DCM, methanol, and water extracts. Then fractionated using colum chromatography led to collect fraction A with IC₅₀ value; 298,74 µg/mL, fraction B; IC₅₀ value 103.73 µg/mL and fraction C; IC₅₀ value 45.41 µg/mL. The fraction C showed highest antioxidant activity compared to fraction A and B which contained tannin and phenolic may have a new lead compound for the development of antioxidants with a novel mechanism of action as an alternative raw material for phytopharmaceuticals.

Keywords: Antioxidant, fractionate, orchid, radical scavengers.



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Introduction

Degenerative diseases, such as hypertension, diabetes mellitus, stroke, and heart disease, are nowadays responsible for 73% of total mortality in Indonesia (Muharram et al., 2024). These diseases were linked to oxidative stress caused by increased free radicals. Reactive oxygen species (ROS) played an important role in cellular tissues caused by UV radiation, cigarette smoke, and environmental stress led to bring about oxidative damage to lipids, proteins, and DNA, raising the risk of degenerative diseases such as cancer, chronic inflammation, neurological disorders, and premature aging (Ansari et al., 2025). The imbalance of free radical production and the body's ability to neutralize free radicals are a major element in the development of pathogenesis. Therefore is needed a novel radical scavengers which is able to neutralize free radicals via electron-donating processes (Chaudhary et al., 2023).

The body's endogenous antioxidant system, which includes superoxide dismutase, catalase, and glutathione peroxidase, has a limited ability to protect the skin from severe oxidative stress, so the body may require exogenous antioxidant support from outside sources, such as vitamins, polyphenols, flavonoids, carotenoids, and others (Chaudhary et al., 2023; Karczowska-Lacka et al.,

2023). Antioxidants function through a variety of mechanisms, including electron transfer (single electron transfer), hydrogen atom donation (hydrog), metal ion chelation to prevent further oxidative reactions, and activation of cellular defense pathways such as Nrf2, which increases antioxidant enzyme expression (Chaudhary et al., 2023). Thus, the presence of exogenous antioxidants is critical for reducing oxidative stress and protecting tissues from further damage (Bakheit et al., 2024).

The search for a new potential of natural products as a source of exogenous antioxidants is very urgent to be studied considering that commercially available antioxidants cannot be relied upon to reduce the prevalence and incidence of exposure to UV rays, mutagenic and other dangerous pollutants. Previous study has shown that the Squirrel Tail orchid contains bioactive secondary metabolites such as phenolics and flavonoids, which function as antioxidants. Noorjahan et al (2024) reported that the root of the ethyl acetate extract was discovered to include alkaloids, flavonoids, phenolics, tannins, and terpenoids, as well as the phenol compound 2,4-bis[1,1-dimethylethyl]-, which is known as a free radical scavenger. Kumari et al. (2024) found that the ethyl acetate fraction had the highest antioxidant activity, with an IC₅₀ value of 36 µg/mL. This suggests a link between high phenolic-

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flavonoid content and free radical scavenging. However, investigations on the antioxidant activity of *R. retusa* root fractionates have not been thoroughly explored, thus additional separation is required to identify putative active constituents using the DPPH test (Kumari et al., 2024.; Noorjahan et al., 2024).

Flavonoids and phenolics are secondary metabolite that have a high antioxidant activity due to their chemical structure's capacity to destroy free radicals. Flavonoids work by inhibiting and neutralizing reactive oxygen species (ROS) via their hydroxyl groups and conjugated double bonds (Zahra et al., 2024). Phenolics act by contributing hydrogen or electrons to create stable phenoxyl radicals, which block oxidative chain reactions (Gulcin, 2025). Based on preliminary tests and other investigation, it showed that the roots of the squirrel tail orchid may contain new sources of antioxidants as a lead compound with specific mechanism to increase the expression of the body's endogenous antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, which play an important role in protecting the skin from free radical

Methods

Materials

The instruments used in this study comprised a drying oven, grinder, rotary evaporator, chromatography column, glassware, UV lamps at 254 nm and 366 nm, sonicator, pycnometer, as well as a UV-Vis spectrophotometer and a microplate reader (Thermo Scientific Multiskan FC 357 Microplate Photometer).

The plant materials included roots of the squirrel tail orchid (*R. retusa*) obtained from the Kayu Jati Hamlet Forest, Malang, as well as technical-grade solvents such as methanol (MeOH), *n*-hexane, ethyl acetate (AcOEt), dichloromethane (DCM), and distilled water for extraction and fractionation purposes. The phytochemical test reagents used were Dragendorff, Mayer, and Wagner reagents, HCl, H₂SO₄, chloroform, Liebermann-Burchard, magnesium powder, 10% NaOH, 1% FeCl₃, 1% gelatin, 1% NaCl and KMnO₄ with standards such as gallic acid, tannic acid, quercetin, pure saponin, beta-sitosterol and caffeine as reference compounds. For antioxidant analysis, used DPPH (Sigma-Aldrich) and ascorbate acid were utilized as positive controls.

Determination and Preparation of Simplicia

The squirrel tail orchid was identified taxonomically at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, to ensure its scientific designation. Preparation of the root simplicia involved wet sorting to remove impurities and unused parts, followed by slicing and oven-drying at 40–50 °C until an appropriate moisture content was achieved. The dried samples were then to dry sorted, pulverized using a grinder, and weighed. The resulting simplicia powder was stored in a clean, closed container, and its yield was then calculated.

Extraction and Fractionation of Active Substances

The simplicia powder from squirrel tail orchid roots (*R. retusa*) was defatted. A total of 300 g of the powder was first macerated with 500 mL of *n*-hexane for 24 hours with periodic agitation for three cycles to remove nonpolar components in room temperature. The residue was then extracted with methanol; the extract was filtered and evaporated, after which the residue was re-extracted with methanol. All macerates were filtered and

concentrated using a rotary evaporator to obtain a viscous extract. The residue containing active component was subsequently refluxed with 250 mL of methanol at 60 °C for 2 hours, repeated three times. The resulting reflux filtrates were evaporated to yield a viscous extract, and the extraction yield was then calculated.

Phytochemical screening of extracts and the active fractionate

Triterpenoid/steroid screening was performed using the Liebermann-Burchard and Salkowski tests. In the Liebermann-Burchard test, 1 mL of chloroform was applied to the sample, followed by the addition of three drops of Liebermann-Burchard reagent. A reddish-brown color indicated a positive result for triterpenoids, whereas a blue-green color indicated steroids (Wichayapreechar et al., 2025). The results were further confirmed using the Salkowski test, in which the sample was treated with 1 mL of chloroform and 1 mL of H₂SO₄. The greenish yellow color change indicated a positive result for steroids, while a reddish brown change indicated terpenoids (Godlewska et al., 2022).

Alkaloid testing involved adding 2 ml HCl to the sample, stirring it, and then adding 2 ml of Dragendorff reagent. The formation of a reddish brown precipitate indicated a positive result. Confirmation was performed using Mayer and Wagner reagents with the same method, where a brown precipitate (Wagner) indicated positive results (Faruq et al., 2024) and a white or yellowish white colored precipitate (Mayer) positive results (Zatla & Hammoudi, 2024).

Phenolic compounds were tested by adding 2 drops of FeCl₃ to 1 mL of the sample; a color change to blackish blue indicates a positive result (Shaikh et al., 2024). The KMnO₄ reagent was used to confirm the affirmative result, which was shown by decolorization of the solution (Jeevan et al., 2019).

Flavonoid testing was carried out by adding 2–4 drops of 10% NaOH to the sample. A yellow to brownish yellow tint indicated a positive result (Gazali et al., 2023). In order to confirm the result was carried out by adding 2 drops of concentrated HCl and magnesium powder; the formation red or orange colored foam or effervescence indicated a positive result (Zatla & Hammoudi, 2024).

Tannin testing included mixing the sample with distilled water, then adding 1 ml of 1% FeCl₃ to a 1 mL solution. The formation of a blackish blue, greenish brown or greenish blue coloration indicated a positive result (Zatla & Hammoudi, 2024). The test was also carried out by adding 1% gelatin and 1% NaCl, and the production of a white precipitate indicated a positive result (Khan et al., 2023).

Saponin testing was carried out by adding 0.5 sample with 5 ml aquadest to the sample and shaking vigorously. The presence of sustained foam indicated a positive result for saponins (Itam et al., 2021). The results were further validated by the addition of Liebermann-Burchard reagent, where the production of a brown or violet ring indicated the presence of triterpenoid saponins, while a green or blue coloration showed the presence of steroidal saponins (Rafik et al., 2021).

Fractination

Before being loaded onto the chromatography column, 0.5 g of the active ethyl acetate extract was impregnated with silica gel 60. The column was pre-equilibrated with the selected solvent

system, and fractionation was accomplished using a gradient elution of DCM:MeOH:H₂O at 30:3:1, 15:3:1, and 3:3:1, respectively. Each collected fraction was then subjected to thin-layer chromatography to determine secondary metabolite profiles and purity. The usage of a stepwise solvent system is based on polarity differences, with DCM establishing the elution of semi-polar molecules and methanol and water extracting more polar components. This method allows for more systematic separation of fractions based on polarity, hence increasing the efficiency of bioactive fractionate purification.

Evaluation of antioxidant activity

The DPPH solution was produced at a concentration of 0.02 mM. The initial 125 ppm sample was gradually diluted to half its concentration: 62.5 ppm, 31.25 ppm, and 15.625 ppm. A 100 μ L sample of each concentration was added to a microplate well, followed by 100 μ L of 0.02 mM DPPH solution. The mixture was incubated in the dark for 30 minutes before being tested for absorbance with a Thermo Scientific Multiskan FC 357 Microplate Photometer. The decrease in absorbance was used to compute percent inhibition as compared to the DPPH control (Tamfu et al., 2020)

Result and Discussion

Padjajaran University's Plant Taxonomy Laboratory identified the squirrel tail orchid as *Rhynchostylis retusa*. A 575-gram sample of orchid plant was pulverized into a basic powder, offering up to 105 grams (18.26%), with the remaining 470 grams (81.74%) containing components that could not be further processed. The simplicial powder macerated and refluxed with methanol gave 35 grams (33.5%) of methanol extract, while the remaining 70 grams (66.5%) were components that could not be used in later extraction processes. The methanol extract was then partitioned with n-hexane, resulting in 5.4 grams (15.5%), 13.2 grams (37.7%), 6.9 grams (20%), and 9.3 grams (26.8%), as indicated in Table 2. The ethyl acetate extract presented the highest antioxidant activity, with an IC₅₀ value of 33.47 μ g/mL, as shown in Table 3. Following phytochemical screening of the tested extracts, it was observed that the ethyl acetate extract contains secondary metabolites such as alkaloids, flavonoids, phenolics, and tannins, as illustrated in Table 4. To determine the antioxidant potential, 0.5 grams of ethyl acetate extract with antioxidant potential was fractionated using a chromatography column, yielding three active fractions: fractions A, B, and C, with fraction A weighing 16 mg (3.2%), fraction B 23 mg (4.6%), and fraction C 59 mg (19.6%), and the remaining 402 mg (80.4%) were components that could not be fractionated. Fraction A had the highest antioxidant activity (IC₅₀ = 298.74 μ g/mL), followed by fraction B (103.73 μ g/mL) and fraction C (45.41 μ g/mL). Ascorbic acid was utilized as a standard reference standard with an IC₅₀ value of 10.82 μ g/mL, which is shown in Table 5. To confirm the fractions' potential antioxidant activity, different stain patterns were obtained with R_f values of 0.45, 0.31, and 0.14, as shown in Figure 1.

The foxtail orchid roots identified as *Rhynchostylis retusa* exhibit morphological traits compatible with those described in the literature, notably monopodial epiphytic growth with connected, absorbing, and hanging roots. The aerial roots are thick, cylindrical, green, and covered in a velamen layer, which allows

for effective moisture and nutrient absorption. The stem is short and inflexible, but the leaves are distichous, arching, dark green, meaty, leathery, and thick (Rajan et al., 2025)

The yield value of only 18.26% in this study demonstrates the effectiveness of obtaining simplicia powder, which is important for determining the representative antioxidant potential of the *R. retusa* plant. The remaining 81.74% is a volatile component, liquid, sap, cell fluid, mukilagi (mucus), resin, and other components that are not required (Babarabie et al., 2025; Cassola et al., 2019).

The accumulation of active antioxidant content found in the 33.5% methanol extract is an important target for the discovery of potential antioxidants, considering that the methanol extract contains active antioxidant ingredients that are conducive to human cell tissue and contain polar active components that can interact with the active components of the methanol extract of the roots of *R. retusa*, while the accumulation of 66.5% of the non-polar powder of the simplicia (Plaskova & Mlcek, 2023).

The active components of the methanol extract were divided into extracts of varied degrees of polarity, from non-polar to polar. Table 3 demonstrates that the non-polar extract of n-hexane has antioxidant activity, with an IC₅₀ value of 88.58 μ g/mL. The semi-polar extract, ethyl acetate, has an IC₅₀ value of 33.47 μ g/mL, which increases more than twice as polarity increases compared to the n-hexane extract. The dichloromethane extract, which is somewhat more polar than ethyl acetate, exhibited an IC₅₀ value of 75.60 μ g/mL, indicating a 2-fold decrease in antioxidant activity. The IC₅₀ value of the quite polar extract, water, was 176.08 μ g/mL, indicating insufficient antioxidant action. According to these data, the semi-polar ethyl acetate extract obtained the most antioxidant content, showing that the *R. retusa* root sample had the greatest potential for antioxidant activity in the relatively semi-polar extract (Landa-Moreno et al., 2023; Wang et al., 2025).

The phytochemical screening results, as shown in Table 4, show that the ethyl acetate extract, which has been proven to have antioxidant activity, contains phenolics, flavonoids, and tannins, whereas the other extracts contain alkaloids, steroids, and terpenoids, indicating a low contribution as an antioxidant with a weak and non-specific interaction with the target antioxidant receptor (González-Espíndola et al., 2024).

The results of fractionation of ethyl acetate extract reveal that the more polar the fractionate, the higher the active component content. This condition demonstrates that the separation process using chromatography techniques with silica gel as a stationary phase was successful in significantly fractionating non-polar components and polar components with successive polarity levels using eluents DCM:MeOH:H₂O 30:3:1, 15:3:1, and 3:3:1, where only components with a specific affinity for the stationary phase and mobile phase can be eluted and separated effectively.

As seen in Table 5, the more polar fractionates showed higher antioxidant activity. Fraction C exhibits higher antioxidant activity (IC₅₀ = 45.41 μ g/mL) than fractions A and B. Fractionate B exhibits nearly thrice the antioxidant activity of fractionate A (IC₅₀ = 103.73 μ g/mL). These facts are directly related to the fractionate yield, as shown in Table 4. Fractionate C had 4 times lower antioxidant activity than the positive control (ascorbic acid), with an IC₅₀ value of 10.82 μ g/mL.

The higher polarity level of the ethyl acetate fractionate indicates its high antioxidant activity, as shown in Table 5. This is also supported by the stain pattern seen in the chromatogram of

figure 1, which shows that, in addition to the purity level of each fractionate, the R_f value varies greatly between A, B, and C, with the higher polarity level of the fractionate (Fioroni et al., 2023).

Table 1. Results of Extraction of Simplicial Powder with Methanol

Weight of Simplicia (grams)	Crude Extract Weight (grams)	%Extract Yield
105	35	33,5

Table 2. Yield of Liquid-Liquid Extraction Results of Squirrel Tail Orchid Roots

Solvent	Total Volume (ml)	Total Weight (g)	%Yield
<i>n</i> -Hexane	7	5,4	15,5
Ethyl Acetate	14	13,1	37,7
Dichloromethane	6	7	20
Water	10	9,3	26,8

Table 3. Results of Evaluation of Antioxidant Activity of Methanol Extract and Liquid-Liquid Extraction

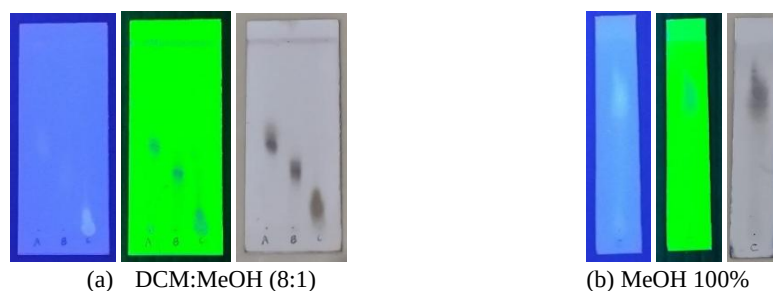
Extract	IC ₅₀ Value (ug/ml)
Squirrel Tail Orchid Roots	
Crude MeOH	185.59
<i>n</i> -Hexane	88.58
Ethyl Acetate	33.47
Dichloromethane	75.60
Water	176.08
Positive Control (Ascorbic Acid)	10,82

Table 4. Phytochemical Screening Results of Squirrel Tail Orchid Roots

Metabolite Group	Reagent	Liquid-Liquid Extract Screening Results					Fraction Screening Results		
		Crude MeOH	<i>n</i> -Hexane	Ethyl Acetate	DC M	Water	Faction A	Faction B	Faction C
Alkaloid	Wagner	+	+	-	+	+	+	-	-
	Mayer	+	+	+	-	-	-	-	-
	Dragendorff	+	+	+	+	+	+	-	-
Phenolic	FeCl ₃ 1%	+	-	+	+	+	-	-	+
	KMnO ₄	+	-	+	+	+	-	-	+
Flavonoid	HCl+Mg	+	-	+	-	+	-	+	-
	NaOH 10%	+	-	+	+	+	-	+	-
Saponin	H ₂ O, HCl 2N	-	-	-	-	-	-	-	-
	Lieberman Burchard	-	-	-	-	-	-	-	-
Tannin	Gelatin 1%+NaCl 1%	-	-	+	-	-	-	-	+
	FeCl ₃ 1%	+	-	+	+	-	-	-	+
Steroid	As.Asetat+ H ₂ SO ₄	-	+	-	+	-	-	-	-
	Salkowski	-	-	-	-	-	-	-	-
Terpenoid	As.Asetat+ H ₂ SO ₄	-	-	-	-	-	-	-	-
	Salkowski	-	-	-	-	-	-	-	-

Table 5. Results of Antioxidant Activity Evaluation on Fractionates

Sample	IC ₅₀ Value (ug/ml)
Fractionate A	298,74
Fractionate B	103,73
Fractionate C	45,41
Ascorbic Acid	10,82

**Figure 1. Results of Chromatograms of Fractionates A, B, and C**

Conclusion:

The ethyl acetate extract and fractionate of squirrel tail orchid roots demonstrated considerable antioxidant activity. The ethyl acetate extract has an IC₅₀ value of 33.47 µg/mL, indicating a strong antioxidant. The active fractionates showed decreased bioactivity, with Fractionate B having an IC₅₀ value of 103.73 µg/mL and Fractionate C having an IC₅₀ value of 45.41 µg/mL, compared to the positive control's IC₅₀ value of 10.82 µg/mL. Both fractionates contain active components such as phenols, tannins, and flavonoids. The results obtained suggest that *R. retusa* roots contain antioxidants and bioactive components.

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