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Phytochemical Screening and Antioxidant Potential of Solvent Fractions from the Stem Bark of *Zanthoxylum rhetsa* (Roxb.) DC.

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Abstract

Zanthoxylum rhetsa (Roxb.) DC. or also known as Indian Prickly Ash is one of Indonesian plant that has broad application in daily life. Wood is used as furniture and fruit is often used as spice or directly eaten. Several studies have shown that the stem bark of *Z. rhetsa* consists mainly of alkaloid and lignan. The purpose of this study was to analyze the phytochemical and antioxidant properties of several extracts and fractions of the stem bark of *Z. rhetsa*. The Methanol extract derived from maceration process was partitioned using *n*-hexane, ethyl acetate, and *n*-butanol. Functional group identification was performed using FTIR (Fourier transform infrared). DPPH (1,1-diphenyl-2-picrylhydrazyl) assay at a wavelength of 517 nm was used to measure antioxidant activity. The method was validated using ascorbic acid beforehand. Phytochemical screening included the test for alkaloid, flavonoid, tannin, steroid, and terpenoid, resulting that *n*-hexane fraction showed positive result for terpenoid, steroid, and alkaloid meanwhile ethyl acetate and *n*-butanol fraction showed positive result for alkaloid, flavonoid, and tannin. The result of DPPH assay demonstrated that ethyl acetate fraction possessed the best result and classified as a strong antioxidant agent (IC_{50}=32,109 µg/mL). These results indicate that the ethyl acetate fraction of *Z. rhetsa* stem bark has a great potential as antioxidant agent.

Keywords: antioxidant, DPPH, phytochemical screening, stem bark, *Zanthoxylum rhetsa*

1. INTRODUCTION

Indonesia is given the title of the most diverse country in terms of land and sea biodiversity (National Geographic Indonesia, 2019). As a tropical country, Indonesia has a warm climate with a clear rainfall cycle, which is suitable for plant productivity. Almost 31.750 plant species have been discovered and identified until 2017 (Retnowati et al., 2019). One of the interesting species to review is *panggal buaya* or Indian Prickly-Ash (*Zanthoxylum rhetsa* (Roxb.) DC.).

Panggal buaya is commonly found in Java, Nusa Tenggara, Bali, and Sulawesi. Belong to the Rutaceae family, *Z. rhetsa* is known for having big cone-shaped thorns all over the surface of its stem bark notably on the older tree. People of Bali used wood as a material in making furniture and statues due to its strong and easy-to-carve property (Hardiyanto, 2008; Nhiem et al., 2021). Aside from its unique

morphology, *Z. rhetsa* was practically used as food and medicine in daily life. Leaf and seed from the plant were used in treating asthma, coughing, and fever. The decoction from its leaves was useful for intestinal worms also as an insect repellent (Maduka and Ikpa, 2021). Meanwhile the fruit part was often used as spice or food source (Aziz et al., 2022; Wongkattiya et al., 2018). As for the stem bark, it has been tested active as antibacterial, antifungal, and cytotoxic (Tuyen et al., 2023; Kyaw et al., 2020).

These broad applications of *Z. rhetsa* are due to the rich secondary metabolite contents in the plant. Zanthoxylum species, especially the stem bark, were reported mainly composed by alkaloid and lignan. Phytochemical screening showed that Zanthoxylum species also consist of flavonoid, sterol, and terpenoid (Nhiem et al., 2021). Several studies have been done in order to study secondary metabolite contents and the bioactivities of *Z. rhetsa* in different locations. In 2023, Tuyen et al. succeeded in isolating four different lignans and three non-alkaloids including the new asarinin and horsfieldin from *Z. rhetsa* taken from Son La, Vietnam. One of them was selectively tested as cytotoxic agent (Tuyen et al., 2023). Five alkaloids and one lignan also isolated from methanol extract from *Z. rhetsa* from Narsingdi, Bangladesh with zanthodioline, oxynitidine, fagaridine, and lignan pluviatilol were introduced for the first time (Zohora et al., 2018). Lignan (+)-piperitol-γ,γ-dimethylallylether was derived in the process of isolation using *Z. rhetsa* from Bogor, Indonesia. In similar study, three sinapyl alcohols were also isolated and possessed anticancer property (Ruchiyat et al., 2022; Ruchiyat et al., 2022).

Despite of the abundance, the study of *Z. rhetsa* was rarely performed in Indonesia. Plant samples used in the previous studies were mostly derived from ex-situ conservation sites such as Bogor Botanical Garden and Bali Botanical Garden. As of today, there is no research regarding *Z. rhetsa* that used plant samples from its original habitat, moreover from Sukabumi, West Java. Based on those narratives, it is important to perform a study regarding phytochemical and antioxidant properties of the stem bark of *Z. rhetsa* from Sukabumi, West Java as a preliminary study.

2. MATERIAL AND METHODS

The stem bark used was collected from Buniwangi, Surade subdistrict, Sukabumi, West Java in April and May 2023. After washed and dried using indirect sunlight, all the samples were crushed into powder in PT. Berkah Alam Nusantara, Pangatikan subdistrict, Garut, West Java.

1.342 kg sample powder was extracted with technical-grade methanol (8.5 L) in room temperature within 3 × 72 hours with occasionally stirring. The extract was then evaporated to yield 99.21 g residue of the concentrated extract. The Methanol extract derived from maceration process (50 g) was dissolved in water/methanol (4:1) mixture then went through fractionation by liquid-liquid extraction method started from the highest polarity solvent using technical-grade *n*-hexane, ethyl acetate, and *n*-butanol respectively.

The fraction was done using 175 mL solvent within 6 days each. Phytochemical screening was done by testing 2-3 mL methanol extract and each of the fractions using a specific reagent: technical-grade chloroform and sulphuric acid for terpenoid, Liebermann-Burchard for steroid, Dragendorff (solution A of 0.5 g bismuth nitrate in 10 mL concentrated HCl reacted with solution B of 4 g potassium iodide in aquadest) for alkaloid, FeCl 5% for tannin, NaOH 2% and HCl 1 M for flavonoid.

Functional group identification was performed using FTIR (Thermo Fisher Nicolet 380). DPPH (1,1-diphenyl-2-picrylhydrazyl) assay at 517 nm wavelength (UV-Vis Genesys) was used in measuring antioxidant activity. The method was validated using ascorbic acid beforehand. Further analysis was performed using thin layer chromatography using Silica gel 60 F-254 (Merck) with *n*-hexane/ethyl acetate 7:3 eluent combination.

3. RESULTS AND DISCUSSION

The extraction method used in this study was maceration performed in a temperature room. The container itself was placed in a dark area so it wouldn't be bothered by the sunlight. Before maceration was performed, stem bark sample was crushed into powder in order to broaden its surface area so the extraction process would be done effectively. The bigger the surface area, the easier solvent penetration through the sample and the more compounds would be extracted (Azwanida, 2015; Hidayat and Wulandari, 2021). The weakness of this method is that it takes a long time and requires a huge amount of solvent. Therefore, a long time of soaking the sample allowed more targeted compounds to be extracted so that the extractions become more optimal. Another advantage of maceration is that the compounds within the sample won't be spoiled or degraded because it does not involve heating (Susanty and Bachmid, 2016). 4.5 L methanol extract concentrated by evaporating the solvent, yielded 99.21 g with 7.4% yield.

The concentrated methanol extract was then fractionated by liquid-liquid extraction using organic solvents with different polarity and started with the less polar one, resulting *n*-hexane fraction (22.19 g with 0.89 % yield), ethyl acetate fraction (20.87 g with 41.74 % yield), and *n*-butanol fraction (6.69 g with 13.38 % yield). All these fractions along with methanol extract were further tested using specific reagents in order to determine their phytochemical properties, especially in identifying secondary metabolite contents.

Phytochemical screening included the test for alkaloid, flavonoid, tannin, steroid, and terpenoid as the result displayed on Table 1. Dragendorff test for alkaloid showed a positive result for all of the samples tested. Orange precipitate formed as nitrogen from alkaloid reacted with potassium from Dragendorff resulting a potassium-alkaloid complex (Parbuntari et al. 2018). Except for *n*-hexane fraction, the rest of the samples tested displayed a positive result for flavonoid and tannin test. The yellow color in flavonoid

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test was due to degradation of flavone by base such as NaOH resulting yellow colored acetophenon. As for $FeCl_3$ test, tannin which is a polyphenol structure forms a Fe-phenolic complex with Fe^{3+} from $FeCl_3$ resulting deep blue color for hydrolysable tannin and brownish-green color for condensed tannin (Bharudin et al., 2013).

Table 1. Phytochemical screening result

Secondary metabolites	Reagent	Positive result	Result showed			
			Methanol extract	*n*-Hexane fraction	Ethyl acetate fraction	*n*-Butanol fraction
Alkaloid	Dragendorff	Orange precipitate	Orange precipitate (+)	Orange precipitate (+)	Orange precipitate (+)	Orange precipitate (+)
Flavonoid	NaOH, HCl	Yellow after the addition of NaOH, and disappear after the addition of HCl	Yellow after the addition of NaOH, and disappear after the addition of HCl (+)	No color changes (-)	Yellow after the addition of NaOH, and disappear after the addition of HCl (+)	Yellow after the addition of NaOH, and disappear after the addition of HCl (+)
Tannin	$FeCl_3$ 5%	Deep blue or brownish green	Brownish green (+)	No color changes (-)	Brownish green (+)	Brownish green (+)
Steroid	Acetic anhydride, H_2SO_4	Green	Green (+)	Green (+)	Brown (-)	Brown (-)
Terpenoid	Chloroform, H_2SO_4	Brownish ring	Brownish ring (+)	Brownish ring (+)	Black, no ring formed (-)	Black, no ring formed (-)

On the other hand, only *n*-hexane fraction showed positive results for steroid and terpenoid. Steroid test was performed using Liebermann-Burchard method where acetyl derivative was formed in the addition of acetic anhydride went through a reaction with water hydrolyzed by sulphuric acid. Oxidation of the steroid through conjugated double bond forming resulting green color in this test (Sulistyarini et al., 2020). The Salkowski test was used in identifying terpenoid as it showed brownish ring forming as positive result due to reaction between terpenoid with sulphuric acid (Das et al., 2014). The result of phytochemical

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screening showed that the methanol extract contains all of the secondary metabolites tested because both the polar and nonpolar compounds because it acts as universal solvents. Meanwhile the fractions derived from liquid-liquid extraction only contain secondary metabolites with similar polarity as the solvent.

Further analysis was done by identifying the functional group as the data shown as infrared spectrums above. Interpretation of the spectrum was based on phytochemical screening, which focused on the specific functional group of each secondary metabolite had shown positive results. The spectrum of methanol extract reported N-H stretch peak at 3718.15 cm^{-1}, hydroxyl group at 3373.51 cm^{-1}, and C=C stretch both at 1604.55 cm^{-1} and 1513.51 cm^{-1}.

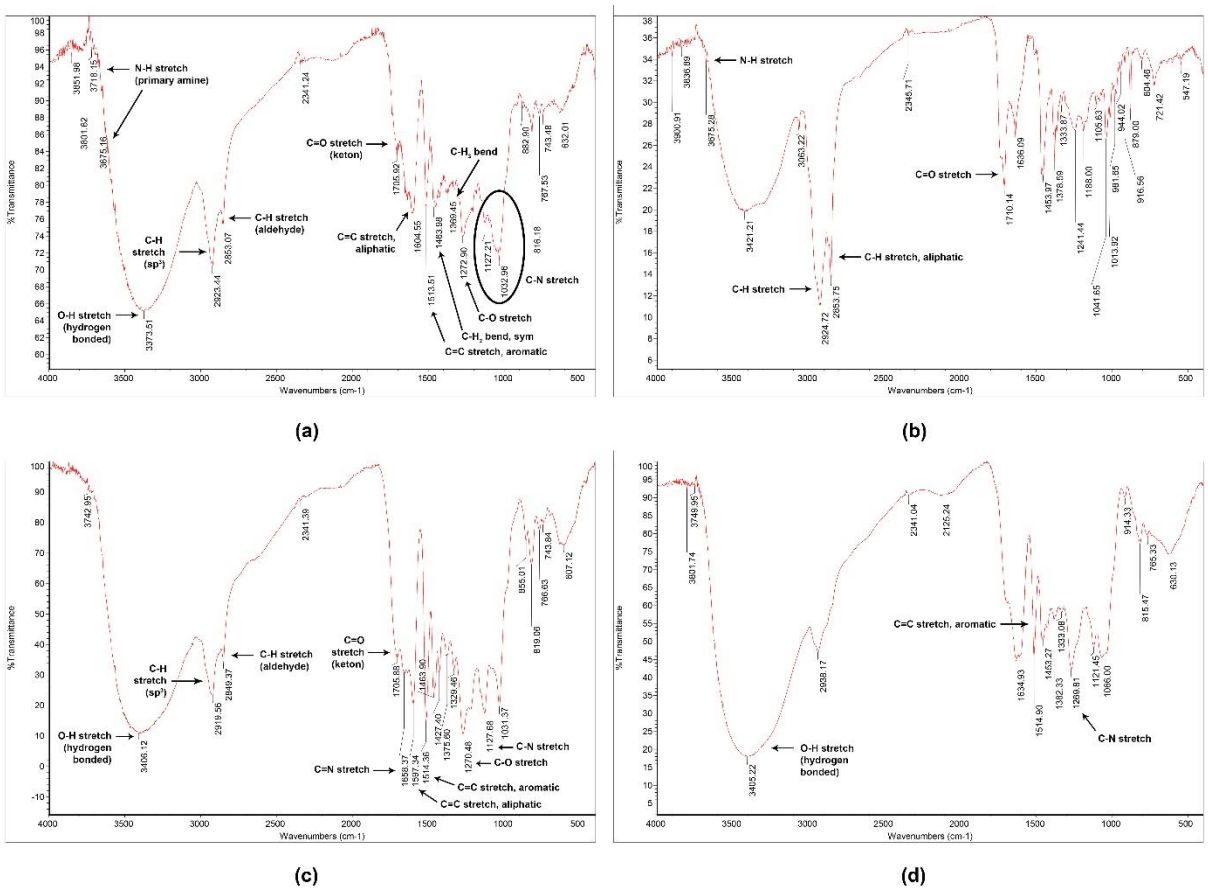

Figure 1. Infrared spectrum of methanol extract (a), *n*-hexane fraction (b), ethyl acetate fraction (c), and *n*-butanol fraction (d)

Those results indicated methanol extract's content of alkaloid, phenolic, and terpenoid. Non-polar compound such as terpenoid and steroid could be identified in *n*-hexane fraction as two strong peaks of C-H group at 2924.72 cm^{-1} and 2853.75 cm^{-1}. As for ethyl acetate fraction, its spectrum displayed a broad and

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strong O-H peak conducting a high phenolic content along with carbonyl and aromatic C=C peaks at 1705.92 cm^{-1} and 1513.51 cm^{-1} implied as flavonoid. The presence of alkaloid is able to be identified as the spectrum showed a strong C-N peak at 1127.68 cm^{-1}. Aside from the carbonyl, *n*-butanol fraction showed a pretty similar result but with lower intensities.

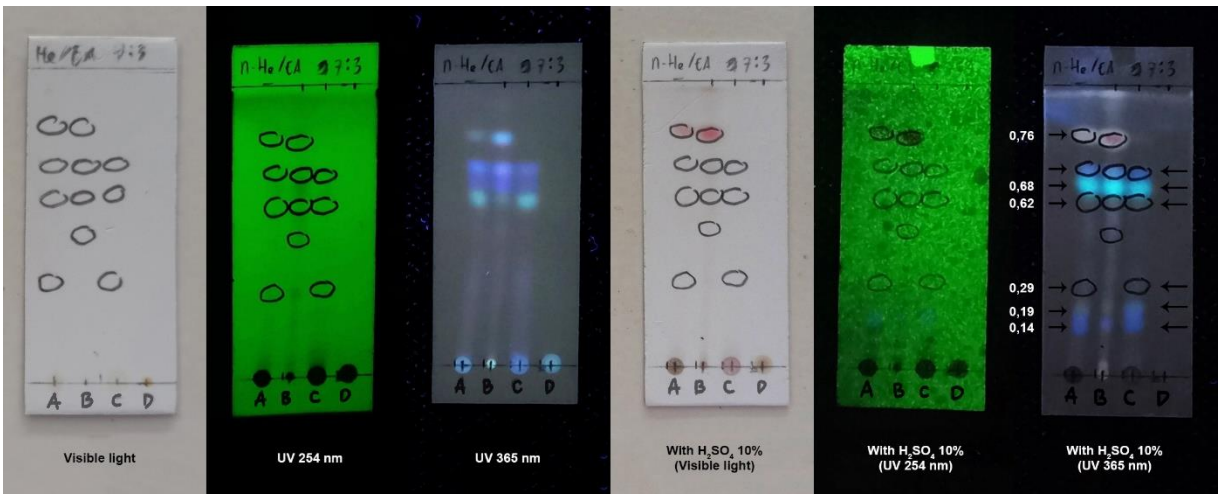

Figure 2. TLC chromatograms contained methanol extract (a), *n*-hexane fraction (b), ethyl acetate fraction (c), and *n*-butanol fraction (d)

Thin layer chromatography was then performed to analyze the component of the extract and fractions derived using silica gel plate as stationary phase and a combination of *n*-hexane/ethyl acetate 7:3 as eluent. Overall, the methanol extract displayed seven different spots. Several spot appeared to be invisible except for the spot left on the base line, which is the more polar compound in the extract and fractions. But after the plate got sprayed by H_2SO_4 10% in ethanol spray reagent, the spot appeared to be more visible and vivid and the brightest spot was the one with Rf value 0.68.

In this study, the antioxidant properties of the samples were tested using DPPH method. The method was validated beforehand using ascorbic acid as standard. Out of all extract and fraction measured, ethyl acetate fraction showed the best result with (IC_{50} = 140 ± 1,20 µg/mL) and *n*-butanol fraction (IC_{50} = 168 ± 0.76 µg/mL) also showed the best result out of methanol, chloroform, and *n*-hexane fraction (Santhanam et al., 2013). The result was prior to the high phenolic content such as flavonoid of ethyl acetate fraction which higher than *n*-butanol one as shown in the FTIR result. Phenolic compounds are one of the strongest natural antioxidants because they consist of an aromatic ring containing one or more hydroxyl substituents and range from simple phenolic molecules to highly polymerized compounds. (Velderrain-Rodríguez et al., 2014; Moo-Huchin et al., 2015).

The phenolic hydroxyl group is directly related to the ability to reduce or inhibit free radicals (Jinxiang et al., 2020; Kaurinovic and V. Djendji, 2019). It could also due to water usage in solvent system in the liquid-liquid fractionation that could possibly lead to flavonoid glycosides hydrolysis. Flavonoid glycosides commonly hydrolyzed and form their respective aglycones as the result. The absence of the sugar moiety of flavonoid glycosides is reported to enhance the antioxidant capacity (Kornpointner et al., 2022).

Table 2. DPPH assay result

Sample	Concentration (µg/mL)	Absorbance	Inhibition %	IC_{50} (µg/mL)
Methanol extract	Blank	0.688		57.113
	20	0.507	0.507	
	40	0.404	0.404	
	60	0.326	0.326	
	80	0.244	0.244	
	100	0.182	0.182	
n-Hexane fraction	Blank	0.819		625.073
	50	0.776	5.250	
	100	0.748	3.651	
	150	0.712	4.815	
	200	0.689	3419	
	250	0.646	6.014	
Ethyl acetate fraction	Blank	0.860		32.109
	10	0.656	23.691	
	20	0.544	36.758	
	30	0.432	49.787	
	40	0.349	59.364	
	50	0.270	68.554	
n-Butanol fraction	Blank	0.819		37.820
	10	0.669	18.356	
	20	0.578	29.426	
	30	0.459	43.956	
	40	0.392	52.177	
	50	0.016	62.556	

It was also mentioned in the previous study that ethyl acetate fraction had the highest total phenolic content (20.47 ± 0.09 mg of GAE/g of plant extract) followed by *n*-butanol (14.14 ± 0.18 mg of GAE/g of plant extract) (Santhanam et al., 2013). Meanwhile, methanol extract displayed an even lower antioxidant properties with IC_{50} value of 57.113 µg/mL due to the presence of inactive compound since it had not been

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separated through fractionation in which affected the IC_{50} value. As for *n*-hexane fraction did not active as antioxidant because lacked of antioxidant active compound such as flavonoid and any other phenolic group.

4. CONCLUSIONS

This present study investigated phytochemical properties of *n*-hexane, ethyl acetate, and *n*-butanol fraction from the stem bark of Z. *rhetsa* and their antioxidant properties using DPPH method. Each of the fractions showed different secondary metabolites content. Terpenoid, steroid, and alkaloid were identified in *n*-hexane fraction, as for ethyl acetate and *n*-butanol fraction showed positive results for alkaloid, tannin, and flavonoid. The result of DPPH assay demonstrated that ethyl acetate fraction possessed the best result due to high phenolic contents as showed in IR spectrum and classified as a strong antioxidant agent (IC_{50}=32.109 µg/mL). These results indicate that the ethyl acetate fraction of *Z. rhetsa* stem bark has a great potential as an antioxidant agent.

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