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ARTICLE

FREE RADICAL INHIBITORY ACTIVITY AND PHYTOCHEMICAL PROPERTIES ETHANOLIC EXTRACT OF *Guazuma ulmifolia* LEAVES AND *Rheum officinale* ROOTS

[*Aktivitas Penghambatan Radikal Bebas dan Kandungan Senyawa Fitokimia pada Ekstrak Etanol Daun Guazuma ulmifolia dan Akar Rheum officinale*]

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ABSRACT

Despite the unique characteristics, the investigation of the antioxidant activity of ethanol extract *of Guazuma ulmifolia* leaves and *Rheum officinale* roots extracted by sonication method and associated with phytochemical content, especially rutin compound content has never been conducted. Therefore, this study aimed to evaluate the antioxidant activity and content of phytochemical compounds in ethanol extracts of *G. ulmifolia* leaves and *R. officinale* roots. Extraction was conducted through the sonication method using absolute ethanol solvents, followed by qualitative phytochemical testing and rutin quantitative analysis with UV-Vis spectrophotometry. Free radical inhibitor activity (antioxidant activity) was measured through the DPPH method. The results showed that extract of *G. ulmifolia* leaves contained alkaloids, flavonoids, tannins, and steroids, while *R. officinale* roots had only flavonoids and tannins. The rutin concentration was higher in *G. ulmifolia* compared to *R. officinale* root. However, the antioxidant activity of *R. officinale* roots (IC_{50} = 21.14±2.17 µg/mL) was higher than extract of *G. ulmifolia* leaves (IC_{50} = 46.05±1.93 µg/mL). The synergistic or antagonistic interaction of phytochemical compounds in the crude extract caused this variation. Consequently, further studies were recommended to identify phytochemical compounds' synergy mechanisms or antagonisms contributing to biological activity.

Keywords: Antioxidants, DPPH, Phytochemicals, Rutin

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ABSTRAK

Penelusuran aktivitas antioksidan ekstrak etanol daun Guazuma ulmifolia dan akar Rheum officinale yang diekstraksi dengan metode sonikasi yang dikaitkan dengan kandungan senyawa fitokimia, secara khusus senyawa rutin belum pernah dilakukan. Penelitian ini bertujuan untuk mengevaluasi aktivitas antioksidan dan kandungan senyawa fitokimia pada ekstrak etanol daun G. ulmifolia dan akar R. Officinale. Ekstraksi dilakukan dengan metode sonikasi menggunakan pelarut etanol absolut, diikuti pengujian fitokimia kualitatif dan analisis kuantitatif rutin menggunakan spektrofotometri UV-Vis. Aktivitas antioksidan diukur melalui metode DPPH. Hasil menunjukkan bahwa ekstrak daun G. ulmifolia mengandung alkaloid, flavonoid, tanin, dan steroid, sedangkan ekstrak akar R. officinale hanya mengandung flavonoid dan tanin. Konsentrasi rutin lebih tinggi pada ekstrak daun G. ulmifolia dibandingkan akar R. officinale. Namun, aktivitas antioksidan ekstrak akar R. officinale (IC_{50} = 21,14±2,17 µg/mL) lebih tinggi dibandingkan daun G. ulmifolia (IC_{50} = 46,05±1,93 µg/mL). Hal ini diperkirakan disebabkan oleh interaksi sinergis atau antagonis senyawa fitokimia dalam ekstrak kasar. Penelitian lebih lanjut diperlukan untuk mengidentifikasi mekanisme sinergisitas atau antagonisme senyawa fitokimia yang berkontribusi terhadap aktivitas biologis.

***Kata kunci**: Antioksidan, DPPH, Fitokimia, Rutin*

INTRODUCTION

Indonesia is among the countries practicing the use of natural ingredients from plants for medicine. The use of plants as traditional medicinal ingredients is based on the hereditary use by ancestors, which has been preserved to the present generation. This plant-based traditional medicine, known as herbal medicine, is mostly preferred by the community to overcome various diseases. Apart from the affordable price, the community believes that natural medicines have fewer side effects. Moreover, certain plants contain phytochemical compounds with pharmacological effects that can be used to treat various diseases (Samtiya *et al.*, 2021)

Among various traditional medicinal plants, Dutch teak (*Guazuma ulmifolia*) is widely distributed across several countries such as Cuba, Honduras, Brazil, Mexico, and Bolivia (Pereira *et al.*, 2019). Leaves of *G. ulmifolia*, the part that is usually applied as a medicinal ingredient (Pereira *et al.*, 2019; Smaradhna *et al.,* 2023). The plant is used as a slimming agent (Rafi *et al.,* 2020). The presence of phytochemical compounds such as flavonoids, phenols, and tannins in this plant *(*Rafi *et al.,* 2020), shows its potential to be developed as an antioxidant candidate. Moreover, these compounds have similar characteristics to anti-obesity drug content, allowing inhibition of the proliferation and differentiation of preadipocyte cells (Nuri *et al.,* 2020). Obesity is involved in increased oxidative stress. This occurs through the formation of superoxide, oxidative phosphorylation and glyceraldehyde autoxidation (Manna & Jain, 2015).

Takami *et al*., (2019) stated that antioxidant compounds can increase carbohydrate and fat metabolism aerobically both when resting and exercising. Besides that, Ramadhansyah *et al*., (2023) stated treatment of ethanol extract of *G. ulmifolia* leaves can reduce the development of coronary artery atherosclerosis. The flavonoid content is predicted to act as an antioxidant, which prevents the adverse effects of oxidative stress on the endothelium structure and inhibits oxidation of low-density lipopolysaccharide (LDL) (Ramadansyah *et al*., 2023). One of the flavonoid compounds that act as antioxidants is rutin (Enogieru *et al*., 2018)

Apart from *G. ulmifolia*, Rhubarb (*Rheum officinale*) is another plant in traditional medicine. This plant originates from China and is widely used to treat various disease (Listyana *et al.,* 2019). In China, *R. officinale* roots have been used for over 2 thousand years as a mild laxative. Dai *et al.,* (2024) stated that *R. officinale* leaves juice contained rutin compounds with a percentage of 2.22%, showing the potential to treat chronic inflammatory bowel disease. Various oxidative stress in the body that are involved as modulators of inflammation can be combated with antioxidants (Xu *et al*., 2024).

Based on the description, this study evaluated the free radical inhibitory activity of *G. ulmifolia* leaves and *R. officinale* roots extracts, to evaluate their antioxidant activity. The leaves simplicia of *G. ulmifolia* leaves and *R. officinale* roots were extracted by sonication method with absolute ethanol solvent due to speed and ability to produce a larger yield (Shen *et al.,* 2023). Information regarding the phytochemical compounds of *G. ulmifolia* leaves and *R. officinale* roots extracted by sonication, and its antioxidant activity has never been done before. According to Annegowda *et al.*, (2012), phytochemical compounds can be obtained using sonication method for extraction in star fruit with high antioxidant activity. In this study, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method carried out an antioxidant activity test. This method was selected because of the relatively stable DPPH free radicals at room temperature conditions (Gulcin & Alwasel, 2023). DPPH was also considered fairly simple and fast compared to other methods (Echegaray *et al.*, 2021).

The quality of medicinal ingredients from natural materials was determined by testing the levels of phytochemical compounds contained in simplicia. The screening of phytochemical compounds was carried out qualitatively to determine the presence or absence of alkaloids, flavonoids, tannins, saponins, triterpenoids, and steroid groups in the extract. This is important to determine the content of phytochemical compound groups that can be extracted using absolute ethanol solvents and the sonication extraction method. Furthermore, rutin phytochemical compound levels were tested in the extract with UV-Vis spectrophotometry. This was performed to compare the levels of rutin compounds and antioxidant activity in both plants, thereby determining their future use as medicinal ingredients.

MATERIALS AND METHODS

Extraction

Simplicia was obtained from PT. Capung Indah Abadi, Magelang. The sample was sun-dried, mashed, and sifted with a mesh sieve of 60. Simplicia was extracted using absolute ethanol solvent by sonication method, with time variations of 30, 45, and 60 minutes, temperature 30-40^{o}C, frequency 37 KHz, and power 100% by degassing method. The sonication results were filtered and the solvent was evaporated to obtain a thick extract.

Qualitative Phytochemical Screening

Alkaloids

The extract was weighed 30 mg, followed by the addition of 10 mL of chloroform, and 3 drops of ammonia. A total of 5 drops of H_2SO_4 2N was added and the solution homogenized with a vortex until 2 layers were formed. The top layer was taken and put into 3 different test tubes, each was dripped with a different test reagent, namely Dragendorf, Wagner, and Mayer. The positive results of each reagent were successively shown by the formation of white, brown, and orange to reddish deposits (To'bungan *et al.*, 2024).

Tannins

Approximately 0.1 grams of extract was weighed, added with 9 mL aquades and the solution was brought to a boil with a hotplate magnetic stirrer. The solution was filtered with the Whattman 41 paper. A total of 5 drops of 10% FeCl were added and positive results were characterized by blue/blackish-green color changes (To'bungan *et al.*, 2024).

Saponins

The extract was weighed at 0.1 grams, followed by the addition of 5 mL aquades, and homogenized for 1 minute with vortex. Positive results were foam ± formed 2-3 cm for 15 minutes (To'bungan *et al.*, 2024).

Triterpenoids and Steroids

The extract was weighed at 0.1 grams, added with 1,5 mL of anhydrous acetate, and 0,5 mL drop of concentrated H_2SO_4. Positive reactions of steroids were obtained when a green color was formed, and positive triterpenoid was indicated by the formation of a red color (To'bungan *et al.*, 2024).

Flavonoid

Approximately 0.1 grams of extract was weighed, added with 100 mL of aquades, and the solution was heated for ±5 minutes to boil. The solution was taken 5 mL, supplemented with 0.1 grams of magnesium powder, 1 mL of concentrated HCl, and 5 mL of amyl alcohol, followed by observation of color change (To'bungan *et al.*, 2024).

Quantitative Test of Rutin Content

Rutin quantitative tests started with creating standard curves by dissolving 25 mg of rutin powder with 2 mL of 96% ethanol (1000 ppm). The solution was made in a concentration series (2.5; 5; 10; 20; 30; 40; 50 ppm), where 0.5 mL was collected and added with 1.5 mL of 96% ethanol, 0.1 mL of 10% AlCl2, 0.1 mL of 1 M sodium acetate, and 2.8 mL of aquadest. Subsequently, incubation was carried out for 30 minutes in dark conditions, followed by absorbance measurement using a spectrophotometer UV-Vis at a wavelength of 415 nm. The standard curve was created by plotting absorbance data against the concentration of rutin compounds. Furthermore, linear regression analysis to obtain the equation of the line and the correlation coefficient (r). The r value approaching 1 indicates that the standard curve is linear and can be used to analyze rutin compounds accurately. The rutin concentration was tested by dissolving the ethanol extract of *G. ulmifolia* leaves in 10 ml of 96% ethanol. A 0.5 mL of extract solution was collected, added with 1.5 mL of sodium acetate, and 2.8 mL of aquades. The solution was incubated in the dark for 30 minutes and absorbance was measured at 415 nm (Kementrian Kesehatan Republik Indonesia, 2017)

Free Radical Inhibition Assay (DPPH Method)

A total of 50 µl of test samples were made with various concentrations, plus 1.0 ml of DPPH 0.4 mM, and 3,950 ml of ethanol. The mixture was homogenized using vortex for 30 minutes, and measured for absorption at a wavelength of 517 nm against the blank (consisting of 50 µL of extract and 4,950 ml of ethanol). Control absorbance measurements consisting of 1.0 ml of DPPH and 4.0 ml of ethanol were also measured. Ascorbic acid was used as a comparison. The concentration giving IC_{50} value was obtained from the extract that provided a percent antioxidant activity of 50% compared to the control through a linear regression line equation (Gulcin & Alwasel, 2023).

Data Analysis

Phytochemical data obtained in this study were analyzed descriptively. Free radical inhibition data and rutin levels were statistically analyzed with SPSS 26. Data on yield percentage, qualitative phytochemical content, rutin levels, and IC_{50} values were followed by descriptive analysis.

RESULTS

Extraction

Extraction with different sonication time lengths produced extracts yield as shown in Table 1. The difference in extraction time affected the percentage of extract yield. The sonication duration of 60 minutes showed the highest percentage of yield in both *G. ulmifolia* and *R. officinale*.

Table 1. Percentage yield of ethanol extract of *G. ulmifolia* leaves and *R. officinale* roots (*Persentase rendemen extract etanol daun G. ulmifolia dan akar R. officinale).*

Sonication time length (minutes) (*Lama waktu sonikasi (menit))*	**Yield** (*rendemen*) **(%)**	
	G. ulmifolia	***R. officinale***
30	6.800	22.620
45	6.820	21.460
60	6.860	31.060

Phytochemical Content

Based on qualitative phytochemical tests, the ethanol extract of *G. ulmifolia* leaves was positive for tannins, steroids, flavonoids, and alkaloids, as shown in Table 2. Meanwhile, for phytochemical compounds saponins and triterpenoids showed negative results. Ethanol extract of *R. officinale* roots was positive for flavonoids and tannins. These results showed common differences in the content of phytochemical compounds.

Table 2. Phytochemical compounds based on phytochemical qualitative assay *(Kandungan senyawa fitokimia berdasarkan uji fitokimia kualitatif).*

Extract (*ekstrak*)	**Phytochemical compounds** (*Senyawa Fitokimia*)						
	Alkaloid			**Flavonoid**	**Saponin**	**Tannin**	**Triterpenoid/ steroid**
	Dragen dorf	**Mayer**	**Wagner**				
G. ulmifolia	+	+	-	+	-	+	Steroid
R. officinale	-	-	-	+	-	+	-

Note: positif (+); negative (-). *(Keterangan: positif (+); negatif (-))*

Rutin level testing on ethanol extracts of *G. ulmifolia* leaves and *R. officinale* roots, began with the creation of a standard curve, as shown in Figure 1. The equation obtained from the standard curve was used for rutin level calculation. The results of the test with a UV-Vis spectrophotometer showed rutin contained more in the ethanol extract of *G. ulmifolia* leaves, as presented in Table 3. The different types of phytochemical compounds contained were influenced by many factors. This suggested that different plant species showed variation in phytochemical compounds.

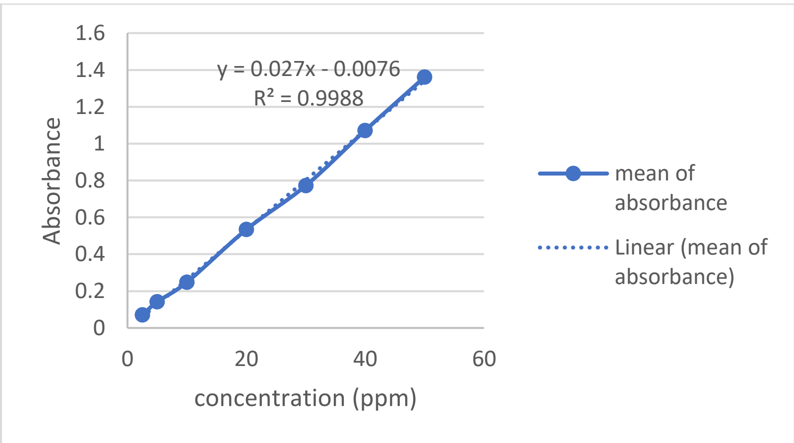

Figure 1. Standard curve of Rutin (*Kurva standard rutin*).

Table 3. Rutin level in *G. ulmifolia* leaves and *R. officinale* roots ethanolic extract at each sonication time (*Kadar rutin pada ekstrak ekstrak etanol daun G. ulmifolia dan akar R. officinale pada masing-masing waktu sonikasi*).

Extract (*Ekstrak*)	**Rutin's levels (mg/g extract)** *(Kadar rutin (mg/g ekstrak)* 30'	45'	60'
G. ulmifolia	43.923±2.530^{b}	46.895± 2.067^{b}	47.295±2.962^{b}
R. officinale	25.620±2.860^{a}	24.297± 3.402^{a}	25.940±2.523^{a}

Note: 30', 45', 60': sonication time 30, 45 and 60 minutes. Superscripts with different letter described the significance (p<0.05) between rutin's level samples (mg/g extract). (*Keterangan: 30', 45', 60': lama waktu sonikasi 30, 45 dan 60 menit. Superskrip dengan huruf yang berbeda menggambarkan signifikansi (p<0,05) antara kadar rutin ekstrak (mgRE/g ekstrak).*

Free Radical Inhibitory Activity

The ethanol extract of *G. ulmifolia* leaves and *R. officinale* roots measured by the DPPH method was with a sonication time of 60 minutes. The IC_{50} value of free radical inhibition in Table 4 showed that the free radical inhibition power *of G. ulmifolia* leaves and *R. officinale* roots ethanolic extract were classified as potential. Meanwhile, ascorbic acid control was in a very potential category.

Table 4. IC_{50} value of ethanolic extract of *G. ulmifolia* leaves of *R. officinale* roots and ascorbic acid *(Nilai IC_{50} ekstrak etanol daun G. ulmifolia dan akar R. officinale serta asam askorbat)*

Extract (*Extract*)	**IC_{50} (ppm)**	**Categories*** (*Kategori*)
G. ulmifolia ethanolic leaves extract *(Ekstrak etanol daun G. ulmifolia)*	46.050±1.933^{c}	Potential (10-50 ppm) *(Potensial (10-50 ppm)*
R. officinale ethanolic roots extract *(Ekstrak etanol akar R. officinale)*	21.140±2.174^{b}	Potential (10-50 ppm) *(Potensial (10-50 ppm)*
Ascorbic acid *(Asam askorbat)*	4.57±1.26^{a}	Highly potent (<10 ppm) *(Sangat potensial (<10 ppm)*

Note: ppm: part per million; IC_{50}: Inhibitory concentration 50; * categories based on (Reviana *et al.*, 2021); superscripts with different letter described the significance (p<0.05) between IC_{50} (ppm) samples.. *(Keterangan: ppm: bagian per seribu; IC_{50}: konsentrasi yang menghambat 50% DPPH; * kategori berdasarkan Reviana et al., 2021; superskrip dengan huruf yang berbeda menggambarkan signifikansi (p<0,05) antara sampel IC_{50} (ppm)).*

DISCUSSION

The use of plants with medicinal properties to overcome certain diseases must be based on scientific studies. Therefore, investigations are carried out to determine the content profile of phytochemical compounds and biological activities. Scientific studies are not only the basis for using medicinal plants but also show future applications. For plants, phytochemical compounds are not directly included in growth (Salam *et al.*, 2023). However, these compounds have an important role in plant defense against stress from the environment (Anulika *et al.*, 2016; Salam *et al.*, 2023). The types of phytochemical compounds contained in plants vary and have specific functions.

In this study, the phytochemical compound test in ethanol extracts of *G. ulmifolia* leaves and *R. officinale* root showed that both had different content. Based on the results, *G. ulmifolia* leaves contained alkaloids, flavonoids, tannins, and steroids, while *R. officinale* roots were only positive for flavonoids and tannins. Variation in results was caused by concentration and different phytochemical compounds in plants due to differences in plant species, environmental factors, and post-harvest handling (Ghasemzadeh *et al.*, 2018; Kumar *et al.*, 2023). According to Hovey *et al.*, (2018) and Adamski et al., (2020), alkaloid compounds are beneficial for defense against predators, with the potential to inhibit the growth of bacteria and fungi. These compounds have benefits for human health including anticancer properties (Adamski *et al.*, 2020), as well as anti-inflammation, cardioprotective (Heinrich *et al.,* 2021). Several classes of alkaloids include isoquinoline, indole, tropane, pyrrolizidine, pyrrolidine, quinolizidine, purine, piperidine, and imidazole. Some of these alkaloids have antioxidant activity that can suppress various diseases by inhibiting free radicals (Thawabteh *et al*., 2019).

Ethanol extract of *G. ulmifolia* leaves was found to contain flavonoids. (Kumar & Pandey, 2013) , mentioned that flavonoids have biological activities such as antioxidants, anti-inflammatory, antiviral and anticancer. In plants, flavonoid compounds act as growth regulators as well as antioxidants (Kumar & Pandey, 2013). Flavonoid compounds act as antioxidants by transferring hydrogen atoms to free radicals (Hassanpour & Doroudi, 2023). The content of this flavonoid compound certainly contributes to the antioxidant activity possessed by *G. ulmifolia* leaf ethanol extract.

In addition to alkaloids, tannins are phytochemical compounds reported to have the potential to overcome various health disorders. Tannins isolated from Cytinus plants show their potential as antioxidants and antibacterial (Maisetta *et al.*, 2019). Atpadkar, Gopavaram and Chaudhary (2023), also mentioned that alkaloids are one of the natural compounds that have antioxidant activity and can be used for health. Alkaloids act as antioxidants by directly warding off free radicals and binding to catalysts involved in a number of oxidation processes (Thawabteh *et al.*, 2019). In addition to being beneficial for health, Ojo, (2022) mentioned that tannins can inhibit the absorption of proteins and minerals. Therefore, the tannin content in *G. ulmifolia* ethanol extract shows the use of the plant's leaves in the community as raw materials for making herbs for slimming.

Steroids are a group of phytosterols in plants supporting growth, development and regulating irritability (Feki, 2023). In addition, Arivarasu (2023), mentioned that one type of phytosterol in plants, namely beta-sitosterol, has significant antioxidant activity in inhibiting hydrogen peroxide and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The steroid content in the ethanol extract of *G. ulmifolia* leaves, is thought to contribute to its antioxidant activity.

Phytochemical compounds detected in both extracts can act as antioxidants. Takami *et al*., (2018) stated that carbohydrate and fat metabolism both at rest and during exercise can be improved through foods rich in antioxidants. Several natural antioxidant compounds have been reported to not only suppress oxidative stress but also obesity. Antioxidants isolated from *Abelmoschus esculentus* L.), namely okra polysaccharides can prevent the increase of free fatty acids, triglycerides and LDL (Yoshitomi *et al*., 2021). In addition, Kukoamine B, which is an alkaloid group, is not only able to prevent inflammation, but also prevent lipid accumulation (Zhao *et al*., 2020). Anyanwu *et al*., (2018) also states that an alkaloid fraction of *Alstonia Boonei*, acts as an antiobesity by increasing the sensitivity of leptin and decreasing the body weight. This activity can occur through various signaling pathways, which still requires further research (Khutami *et al*., 2022)

Flavonoid compounds are phytochemical compounds that are widely reported to act as antioxidants (Speisky *et al.,* 2022). One type of flavonoid glycoside that has antioxidant abilities is rutin (Ganeshpurkar and Saluja, 2017; Enogieru *et al*., 2018). *G. ulmifolia* leaf ethanol extract has a greater rutin concentration than in *R. officinale* root extract, at all sonication incubation times (Table 3).

Rutin which is one of the secondary metabolites of plants is produced to serve as a defense against various environmental stressors (Yeshi *et al*., 2022). It is produced when plants stress, one of which is due to salinity stress (Ismail *et al*., 2016; Zhang *et al*., 2022) This study observed a higher rutin consentration in *G. ulmifolia* extract due to salinity stress intervention experienced by plants in the habitat. In Table 4, the IC_{50} value of ethanol extract treatment of *G. ulmifolia* leaves in the free radical inhibition test showed a larger value (46,050±1,933 μg/mL) compared to *R. officinale* roots (21,140±2,174 μg/mL). This showed that the antioxidant activity of *R. officinale* root extract was higher compared to *G. ulmifolia* leaves extract. Despite the significant differences, the IC_{50} values were still categorized as potential antioxidant (Reviana *et al.,* 2021). This proves that *G. ulmifolia* leaf and *R. officinale* roots extract can be used as a further natural antioxidant candidate. Nevertheless, further research on its safety is still needed.

The concentration of rutin compounds that are not in line with the antioxidant activity of the extract, can be influenced by the composition of phytochemical compounds in the crude extract. The crude extract still contains phytochemical compounds with very diverse compositions and concentrations. Thus, other flavonoid compounds may have antioxidant activity other than the rutin compounds measured in this study. Therefore, the content of other flavonoid compounds, which have the potential to be antioxidants, needs to be measured to support the argument for inconsistent results.

Apart from that, the antagonistic properties of phytochemical compounds may contribute to the incompatibility of free radical inhibitory activity. As shown by qualitative tests, *G. ulmifolia* ethanol extract contained a group of phytochemical compounds more than *R. officinale* roots extract. This was because crude extract that still had complex phytochemical compounds allowed for synergistic and antagonistic effects (Budiyanto *et al.*, 2022). The antagonistic properties are various phytochemical compounds, which can weaken the biological activity of other phytochemical compounds. Sometimes, extract cannot provide the maximum effect when in complex mixtures (Caesar & Cech, 2019). In addition, Milugo *et al.*, 2013 stated that the presence of alkaloids and saponins in the methanol fraction of *Rauvolfia caffra* caused a decrease in its antioxidant activity when compared to the antioxidant activity of each compound. The antagonistic relationship between phytochemicals will affect the efficacy of crude extracts as used in traditional medicine. However, interaction of the phytochemical compound in leaves *G. ulmifolia* and root *R. officinale* extract, certainly still needs further proof.

CONCLUSION

In conclusion, ethanol extract of *G. ulmifolia* leaf and *R. officinale* root showed free radical inhibition ability. This was shown by IC_{50} values of 46.050±1.933 µg/mL and 21.140±2.174 µg/mL, respectively. The ability was supported by the content of phytochemical compounds of the alkaloid group, tannins, flavonoids and steroids. The concentration of rutin was higher in ethanol extract of *G. ulmifolia* leaves. Further research needed to be conducted to investigate the biological activity of each phytochemical compound in ethanol extracts of *G. ulmifolia* leaves and *R. officinale* roots to determine the possible synergy or antagonistic of these phytochemical compounds.

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AUTHOR CONTRIBUTIONS

NT: designed, supervised, collected data, analyzed data and wrote the manuscript. KA: analyzed data and wrote the manuscript. ACY: processed the simplicia, provided technical support and wrote the manuscript.

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