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DETERMINATION OF TOTAL PHENOLIC CONTENT, QUERCETIN, AND RUTIN OF COSMOS CAUDATUS LEAF EXTRACTS AND THEIR CONTRIBUTION TOWARD SCAVENGING DPPH RADICALS

Mustofa Ahda[1,2,3]✉, Amalya Nurul Khairi[2,4], Kholil Fahri[1*], Mohd Salleh Rofiee[5,6]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta
[2]Ahmad Dahlan Halal Center, Universitas Ahmad Dahlan, Yogyakarta
[3]Department of Pharmaceutical Chemistry, International Islamic University Malaysia, Kuantan Malaysia
[4]Department of Food Technology, Faculty of Engineering, Universitas Ahmad Dahlan, Yogyakarta
[5]Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA Selangor Branch, Puncak Alam Campus, Bandar Puncak Alam, Selangor.
[6]Faculty of Health Sciences, Universiti Teknologi MARA Selangor Branch, Puncak Alam Campus, Bandar Puncak Alam, Selangor.
✉mustofa_ahda@yahoo.com

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ABSTRACT

C. caudatus leaves are traditionally served as a salad. The total phenolic content (TPC), rutin and quercetin levels, and inhibition against DPPH radicals were all determined in this study. This plant has a TPC of 35.891-91.321 µg gallic acid equivalent/mg dried extract. Rutin and quercetin levels in this plant are approximately 17.97-18.59 µg/mg and 0.73-0.79 µg/mg, respectively. The extract with the highest TPC and rutin levels is 40% ethanolic extract, whereas the extract with the highest quercetin levels and DPPH radicals inhibition is 80% ethanolic extract. Furthermore, both 80% ethanolic extract and 60% ethanolic extract were classified as being similar in this investigation. Meanwhile, the good solid-to-solvent ratio employed in the extraction is 1:6 (w/v). As the outcome of this research, it was recommended that this herb be extracted using 60-80% ethanolic extract with a solid solvent ratio of 1:6.

1.Introduction

Cosmos caudatus Kunth (*C. caudatus*) leaves have numerous beneficial effects on human health. The main strength of this herb has antioxidant activity (Cheng et al., 2015[a]). It was traditionally eaten as a salad in Asian countries such as Indonesia and Malaysia. Furthermore, this plant can be extracted and used in a variety of pharmaceutical, cosmetic, and food ingredients. The active compound content of herb extracts determines their antioxidant activity. *C. caudatus* leaves have potential bioactive compounds such as ascorbic acid, flavonoids, and their derivates, and chlorogenic acid, (Cheng et al., 2015[a], Mediani et al., 2013; Nurhayati et al., 2018; Seyedreihani, et al., 2016). Research from Chan et al., (2016) reported that its leaves also contain major compounds such as flavonoids, phenolic acids, and diterpenoids. Besides, other active compounds such as α-tocopherol, cyclohexen-1-carboxylic acid, stigmasterol, benzoic acid, lycopene, and myo-inositol are contained in the fresh leaves (Javadi et al., 2014; Javadi et al., 2015). Its leaves also contained α-cadinene as one of the major volatiles among the 13 types of volatiles. In general, these herb leaves contain three types of active compounds such as flavonoids and their derivatives, non-flavonoid

groups, and volatile compound groups (Ahda et al., 2023).

The active compounds in its leaves are primarily responsible for its potency. However, the solvents used can affect the active compounds extracted, resulting in different activities. Therefore, selecting a solvent system is a key technique for producing the desired bioactive compounds, with the polarity of the active compounds being affected by the solvent used (Sasidharan et al., 2011; Altemimi et al., 2017). The hydrophilic compounds can be extracted using polar solvents such as methanol, ethanol, or ethyl-acetate and more lipophilic compounds use dichloromethane, or its mixtures (Sasidharan et al., 2011). Altemimi et al. (2017) reported the sequencing of the solvent polarity, from non-polar to polar is hexane < chloroform < ethylacetate < acetone < methanol < water. Furthermore, the solvent used, such as ethanol, is safer for human consumption, has lower toxicological effects, and is suitable for food systems (Suhaimi et al., 2019).

The effect of solvent was explained by Mediani et al. (2013), who found that an 80% methanol extract of *C. caudatus* had higher antioxidant activity than an 80% ethanol extract. Similarly, Cheng et al. (2016b) found that 100% methanol and 50% ethanol extracts of *C. caudatus* leaves had the highest antioxidant activity and were a powerful solvent for extracting its active compounds when compared to other 100% ethanol, 95% ethanol, and 100% water extracts. Besides the solvent concentration, the solid-to-solvent ratio also affected the extracted active compound from the herbs. Therefore, to evaluate the contribution of quercetin and rutin in the *C. caudatus* leaves, this study looks for the equilibrium state between solid and solvent because the increasing solvent volume will cause an increase in the extracted active compound (Norshazila et al., 2017). Aside from the solvent concentration, the solid-to-solvent ratio influenced the extracted active compound from the herbs. To evaluate the contribution of quercetin and rutin in *C. caudatus* leaves, this study looks for the equilibrium state between solid and solvent in the extraction process.

2. Materials and methods

2.1. Materials

2.1.1. Samples

C. caudatus leaves were collected from West Sembuh, Sidomulyo, Godean, and Yogyakarta.

2.2. Methods

2.2.1. Samples Preparation

This plant was identified by the Department of Biology, Universitas Gadjah Mada. The dried *C. caudatus* leaves were washed using water and dried in Oven at 45 ^{0}C for 4 days. The dried leaves were ground to become a powder in 60 mesh.

2.2.2. Effect of Solvent Concentrations

The extraction procedure using the Soxhlet method was reported by Sharif et al., (2016) with slight modification. A 10 g sample was dissolved in ethanol (1:10, b/v) and then heated at 50 ^{0}C for 3 hours. the sample was filtered and then evaporated at 50 ^{0}C to produce the dried extract. To evaluate solvent concentration, the different concentration of Ethanol were used (100%, 80%, 60%, 40%, and 20%). All dried extracts were determined for the total phenolic content, Flavonoid content, and inhibition activity of DPPH.

2.2.3. Effect of Solid/Solvent Ratio

To evaluate the solid/solvent ratio, leaf extract powder of *C. caudatus* was weighted in variation ratio 1:12.5; 1:10: 1:8; and 1:6 b/v. The extraction procedure follows previous work. A 10 g sample was dissolved in 125 mL ethanol (solid/solvent ratio: 1:12.5, b/v) and then heated at 50 ^{0}C for 3 hours. the sample was filtered and then evaporated at 50 ^{0}C to produce the dried extract. For 1:10; 1:8; 1:6 b/v, 10 g sample was dissolved in 100 ml; 80 ml; 60 mL ethanol, respectively. All dried extracts were determined for the total phenolic content, Flavonoid content, and inhibition activity of DPPH.

2.2.4. Determination of Total Phenolic Content

The determination of total phenolic content (TPC) in the extract of *C. caudatus* leaves following the study reported by Ahda et al.,

(2019) with slight modification. To determine the TPC, 10 mg extract of *C. caudatus* leaves was dissolved using 10 mL water and then reacted with 1.5 mL Folin Ciocalteu (1:10 in water) and mixed for 3 minutes. After that, it was mixed with 1.2 mL of 7.5% sodium carbonate (b/v) and awaited for 60 minutes. The TPC was measured by a UV-Vis spectrophotometer at a maximum lambda of 743 nm. The standard solution used is a gallic acid solution in ranging concentrations from 30-80 µg/mL. The TPC is expressed in µg/mg equivalent of gallic acid.

2.2.5. Determination of Total Flavonoid Content

The determination of flavonoid compound types including rutin and quercetin. A procedure for rutin and quercetin in *C. caudatus* extract using HPLC as reported by Sharifuldin et al., (2016) with slight modification. RP-HPLC Instrument contains a stationary phase using the C18 column (Lichrospher@ 100 (5 µm)) and the mobile phase consists of 2% acetic acid in water and acetonitrile mixtures 70:30 v/v by the isocratic system and the detector used is a UV detector. The flow rate of the mobile phase is controlled at 1 ml/min and detected at 254 nm. The standard solution mixtures of rutin and quercetin were created in ranging of 2.5-80 µg/mL.

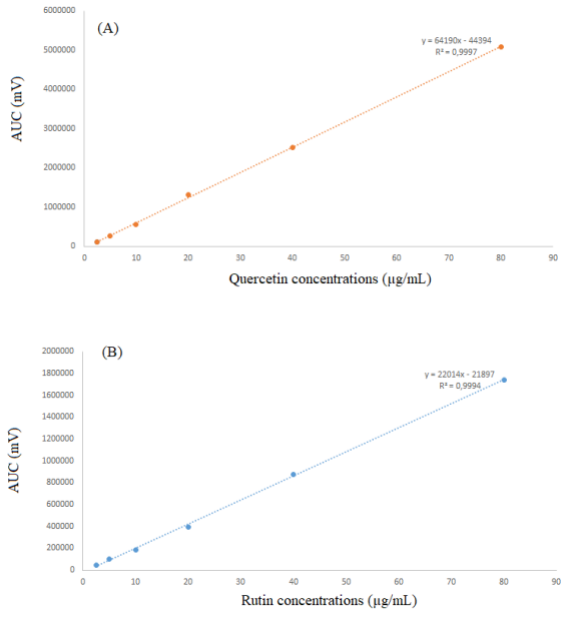

Figure 1. Standard Curve from HPLC: (A) Quercetin Standard; (B) Rutin Standard

Before injection, the sample or extract and standard solutions were filtered using 0.45 syringe filters. The flavonoid and Rutin standard curves (figure 1).

2.2.6. Antioxidant Evaluation of DPPH (2,2-diphenyl-1-picrylhydrazyl) Inhibition

The determination of the antioxidant activity of DPPH was illustrated by Ahda et al., (2019) with slight modification. The extract of *C. caudatus* leaves was dissolved using ethanol in a concentration range of 0-500 µg/mL. 1 mL extract solution was added to 1 mL of 0.05 mM DPPH solution and mixed for 1 minute and then kept for 1 hour. Finally, the solution was read the absorbance by a UV-Vis spectrophotometer at 516 nm and then determine the inhibition concentration 50 (IC_{50}) following the equation:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

Where A_0 is the absorbance of control; A_1 is the absorbance of the samples

2.2.6. Statistic Anlaysis

The data was calculated using Tukey's test to show significant differences ($p<0.05$) and the Coefficient (r) of Person correlation to determine the best correlation between two variables.

3.Results and discussions

3.1. Determination of Quercetin and Rutin Concentrations of *C. caudatus* Leaves extracted by various ethanol concentrations and solid-to-solvent Ratio

Several active compounds in *C. caudatus* leaf extract have high antioxidant activity. This extract contains flavonoids such as quercetin and kaempferol, according to previous research by Andarwulan et al. (2010). In another study, three active compounds were discovered in *C. caudatus* leaf extract: quercitrin, rutin, and quercetin (Sharifuldin, et al., 2016). These flavonoid classes have a strong inhibitory effect on DPPH radicals. However, this study only

examined the levels of rutin and quercetin in the extract.

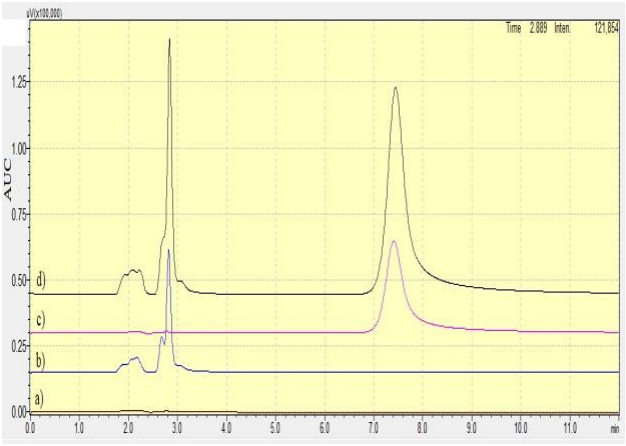

Figure 2. Chromatogram from HPLC (mobile phase: 0.3% formic acid in 0.3% formic acid and acetonitrile mixtures 30:70 v/v): a) Solvent; b) Rutin standard; c) Quercetin standard; d) Mixing of both rutin and quercetin standards

In a previous study, the mobile phase used to detect quercetin and rutin was a mixture of eluent A (water), eluent B (methanol), and eluent C (acetic acid) with gradient technique following initial conditions B 15% and C 5%, then B eluent is increased up to 25% in 15 min, 85% in 5 min, then is kept isocratic for 10 min, and B is decreased up to 15% in 5 min (Iacopini, et al., 2008). This study was carried out for 12 minutes using a mobile phase of 0.3% formic acid: acetonitrile: 30:70 (v/v) with isocratic. Rutin and quercetin were clearly separated, with rutin having a retension time of around 2.8 min and quercetin having a time retention of around 7.3 min, respectively (Figure 2). Figure 3 depicts the presence of four dominant active compounds in *C. caudatus* leaf extract, two of which are rutin and quercetin. Previous research indicates that quercitrin and kaempferol are two active compounds that may be the dominant peak (II) and peak (III). However, in order to clarify this statement, it should be proven by material standards. Based on this result, the mixtures of 0.3% formic acid: acetonitrile: 30:70 (v/v) can separate each component in the leaf extract of *C. caudatus*.

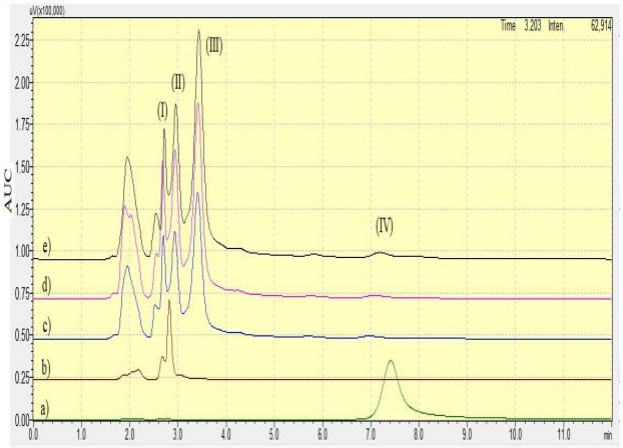

Figure 3. Chromatogram from HPLC (mobile phase: 0.3% formic acid in water and acetonitrile mixtures 30:70 v/v): a) Quercetin standard; b) Rutin standard; c) 40% ethanolic extract of *C. caudatus*; d) 60% ethanolic extract of *C. caudatus*; e) 80% ethanolic extract of *C. caudatus*. (I) rutin; (II) quercetin; (III) and (IV) unknown compounds.

The quercetin and rutin extracted from *C. caudatus* leaves were influenced by solvent concentrations and the solid-to-solvent ratio. According to Ghasemzadeh et al. (2011), increasing the solvent polarity from chloroform to methanol increases the concentrations of quercetin, catechin, and rutin in young Ginger extract. In general, the mechanism of organic solvent with flavonoid and rutin occurs through several steps such as solvation, intermolecular force, and hydrogen bonding interaction (Tamayo-Ramos et al., 2022). As a result, the extracted active compounds are strongly influenced by the extraction process, including solvent concentration, solvent type, temperature, and so on. Furthermore, they are directly associated with biological activities such as antioxidant activity. According to Felhi et al. (2017), solvent extraction affected yield, total phenolic, total flavonoid, and other active compounds, as well as their antioxidant and antimicrobial activities. Based on the current study, the 40% ethanolic extract of *C. caudatus* leaves has the highest rutin concentrations, while the 80% ethanolic extract has the highest quercetin concentrations (Figure 4 A). Besides, the solid-to-solvent ratio affected significantly

the rutin content of this extract but quercetin content was affected slightly. Therefore, in this study, the best solid-to-solvent used is 1:6 or 1:8 based on the rutin content. However, the highest quercetin was produced from the solid-to-solvent ratio of 1:12.5 (Figure 4 B). A previous study reported that both bioactive compounds (rutin and quercetin) play an important role in reducing oxidation damage, with quercetin being more effective than rutin, with DPPH inhibition rates of around 93.8% and 65.3% in Hydroxypropyl -Cyclodextrin formulations, respectively (Basaran et al., 2022). Therefore, the differences in quercetin and rutin contents of *C. caudatus* leaf extract will induce its biological activity including the inhibition activity of DPPH.

3.2. Total Phenolic Content and Inhibition Activity of DPPH Radicals

Total phenolic content (TPC) is a broad parameter that is linked to biological activities such as antioxidant properties. The solvent concentrations used also had an effect on the TPC of the herb extract. According to previous research, 80% methanol and 80% ethanol produced higher TPC and antioxidant activities (Sultana et al., 2009). There is no noticeable difference between TPC extracted with 100% ethanol and TPC extracted with 80% ethanol (Wan-Nadilah et al., 2019). Other studies discovered that extracts containing 50% ethanol, 70% ethanol, and acetone contained the most phenolic and flavonoid compounds, which will contribute to their biological activity as DPPH radical inhibition (Dirar et al., 2018; Ngo et al., 2017). According to Do et al., (2014), 100% ethanol can produce a good extract from *Limnophila aromatica* with TPC value, flavonoid concentrations, and DPPH antioxidant activity of 40.50±0.88 mg gallic acid equivalent/g of defatted extract, 31.11±0.433 mg quercetin equivalent/g of defatted extract, and IC_{50} value of 70.06±1.0 0 mg/mL, respectively.

According to this study, 40% ethanol is a good solvent for producing C. The highest TPC value and Rutin concentrations are found in *C. caudatus* extract, which has 91.32±0.92 µg gallic acid equivalent/mg dried extract and 11.35±0.19 µg/mg, respectively (Figure 5 A and Figure 4 A). Meanwhile, 80% ethanolic extract has the highest quercetin concentrations and DPPH radical inhibition activity (Figure 4 A and Figure 5 B). This study found that rutin content contributed to TPC, whereas quercetin is an active compound that inhibits DPPH radicals more effectively than rutin. The highest antioxidant activity (smaller IC_{50}: 116.66 µg/mL) was demonstrated by the 80% ethanolic extract. A similar result from Iacopini et al., (2008) reported that quercetin has better inhibition activity than rutin where inhibition activities are 22.37±0.3% and 17.17±0.3%, respectively.

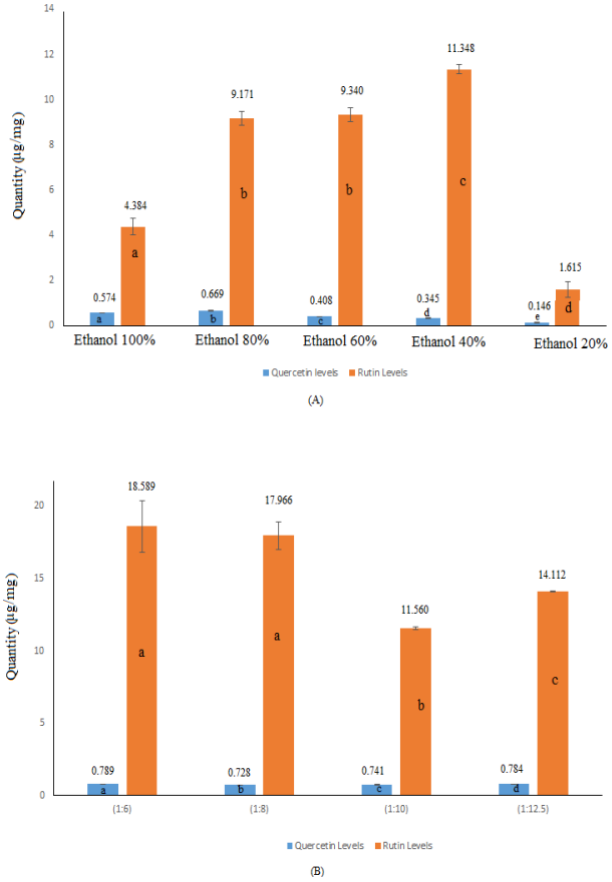

Figure 4. Quercetin and Rutin concentrations of *C. caudatus* leaves extracted by various ethanol concentrations (A) and raw material to ethanol ratio (B). Tukey's tests: a, b, c, d, and e have $p < 0.05$

Aside from solvent concentration, the solid-to-solvent ratio influences the extracted active compound from herbs. Wong et al. (2013) found that a solid-to-solvent ratio of 1:20 had a significant effect on TPC, TFC, and both antioxidant activities of DPPH and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+\cdot}$). Despite this, a higher solid-to-solvent ratio increases the active compound's interaction with the solvent, and thus the extracted active compounds also increase. According to another study, increasing the solid-to-solvent ratio affected the extracted carotenoids from herbs (Norshaliza et al., 2017; Dianursanti et al., 2020).

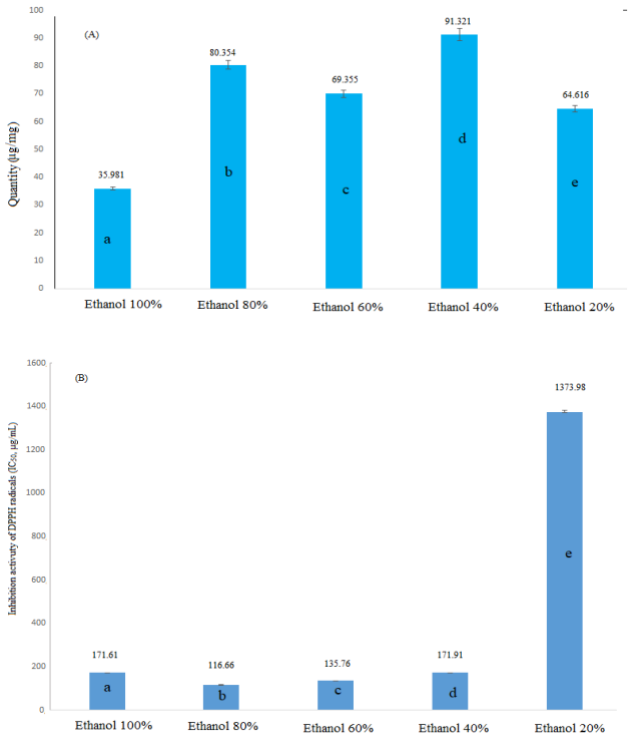

Figure 5. TPC of *C. caudatus* leaves (A) and Inhibition Activity of DPPH (B). n:3; Tukey's tests: a, b, c, d, and e have $p< 0.05$

Figure 6 illustrated that the increase in the solid-to-solvent ratio caused the TPC is decreasing. The optimum solid-to-solvent ratio providing the higher TPC is at 1:6 and 1:8 (Figure 5 A). Besides, the extract of *C. caudatus* (1:6) contains quercetin content is higher than the extract of *C. caudatus* (1:8) but the rutin content is the opposite. Rutin and quercetin concentrations of this herb are about 17.97-18.59 µg/mg (or 1.79-1.86%) and 0.73-0.79 µg/mg (or 0.073-0.079%), respectively. Previous research has reported that rutin and quercetin concentrations in *C. caudatus* are about 0.38-0.94% and 0.26-0.92%, respectively (Sharifuldin et al., 2016; Seyedreihani et al., 2016). However, the solid-to-solvent ratio of 1:6 is the better scavenging activity of DPPH radicals than 1:8 with IC_{50} values are 108.28±0.01 µg/mL and 128.81±0.18 µg/mL, respectively (Figure 5 B). In this case, quercetin content also acts significantly on inhibiting DPPH radicals.

According to this finding, the solid-to-solvent ratio is not always positively correlated with TPC, rutin, and quercetin concentrations, or their antioxidant activity, but is highly dependent on the nature of the active compound taken up by the solvent. According to this study, a smaller solid-to-solvent ratio produces the best extract, which contains more active compounds extracted and its antioxidant activity of DPPH because the kinetic rate of diffusion cannot be controlled for the extraction process at 50 ^{0}C. However, as the solvent used is reduced, the extraction temperature rises, and the extracted active compound rises as well.

In the discriminant analysis, both the 80% and 60% ethanol extracts of *C. caudatus* leaves were significant neighbors (Figure 7). This study discovered that ethanolic extracts of *C. caudatus* leave at 80% and 60% concentrations were not significantly different. This was supported by rutin levels in both the 80% ethanolic extract and the 60% ethanolic extract of this herb. Both extracts effectively inhibit DPPH radicals. It is further supported by Wan-Nadilah et al. (2019), who properly categorised the various extracts of this plant using H-NMR spectroscopy combined with Principal component analysis (PCA), with 60% extract and 80% extract being the closest. This study also produced a classification clarified by PC1 and PC2 of all *C. caudatus* leaf extracts with an Eigenvalue cumulative is 100%. (Figure 7). Therefore, the similarity of *C. caudatus* leaves 80% ethanol extract and 60% ethanol extract is

more accurate. To ensure this study, the determination of all active compounds in *C. caudatus* leaf extracts should be performed using an advanced analytical method such as LC-MS/MS

3.3. The Correlation of TPC, Rutin and Quercetin Levels, and Scavenging Activity of DPPH Radicals

The correlation of active compounds from *C. caudatus* leaves ethanol extract is usually related to TPC because it greatly contributed to its activity, particularly the DPPH radical scavenging activity (Muflihah et al., 2021). The best correlation between TPC and its antioxidant activity will provide useful information for product quality control. Several active compounds from Allium extract, such as catechin, epigallocatechin, and epicatechin gallate, were strongly influenced by antioxidant activity (Beretta et al., 2017). As a result, the concentrations of rutin and quercetin, TPC, and the DPPH radical scavenging activity will be correlated in this study.

This study explained the relationship between the active compounds in *C. caudatus* leaf extract and its antioxidant activity. Quercetin and rutin have a weak correlation with DPPH inhibition (Figure 8 in supplementary data). However, the antioxidant activity of an active compound is significantly affected by its configuration, total number of hydroxyl groups, and substitution of functional groups within the structure (Kumar and Pandey, 2013). Previous research found that curcumin is the most active compound in *Zingiberaceae* species and is highly correlated with antioxidant activity, while quercetin is the inverse (Muflihah et al., 2021). According to Souza et al. (2021), several active compounds in tomatoes, such as anthocyanins and carotenoids, have a moderate correlation with FRAP and a weak correlation with DPPH inhibition. Table 1 shows a Pearson's correlation that supported this finding. According to Table 1. Rutin concentrations correlate better with TPC than quercetin, while quercetin correlates better with DPPH inhibition than rutin. As a consequence, the mechanism of action takes place in this study to further clarify this finding, as illustrated in Figure 9.

The inhibition of DPPH radicals is heavily dependent on the ease of proton radical movement, so the DPPH radical becomes a non-radical product. Shamsudin et al., (2022) reported that the hydroxyl groups in the ring of C5, C7, C3', and C4' will cause antibacterial activity increase. According to its chemical structure, quercetin has 5 hydroxyl groups that are likely to inhibit DPPH radicals, whereas rutin only has 4 hydroxy groups involved in this reaction. The glucoside component of rutin has no effect on DPPH inhibition, but it is highly reactive with the Folin ciocalteu reagent. As a result, quercetin levels correlate with DPPH radical inhibition, while rutin levels correlate with TPC (Table 1). However, the antioxidant activity of quercetin is better than rutin (Kessler et al., 2003; Hernández-Barreto et al., 2023).

Table 1. Coefficient (r) of Pearson's correlation between TPC, Rutin and Quercetin concentrations and scavenging activity of DPPH

Variables	**TPC**	**Scavenging activity of DPPH (IC_{50}, µg/mL)**
TPC	1	-0.428
Rutin concentrations	0.880	-0.632
Quercetin concentrations	0.797	-0.713
Scavenging activity of DPPH	-0.428	1

* Medium Pearson's Correlation ($0.4 < r < 0.59$); high Pearson's Correlation ($0.6 < r < 0.79$); and very high Pearson's Correlation ($0.8 < r < 1.0$)

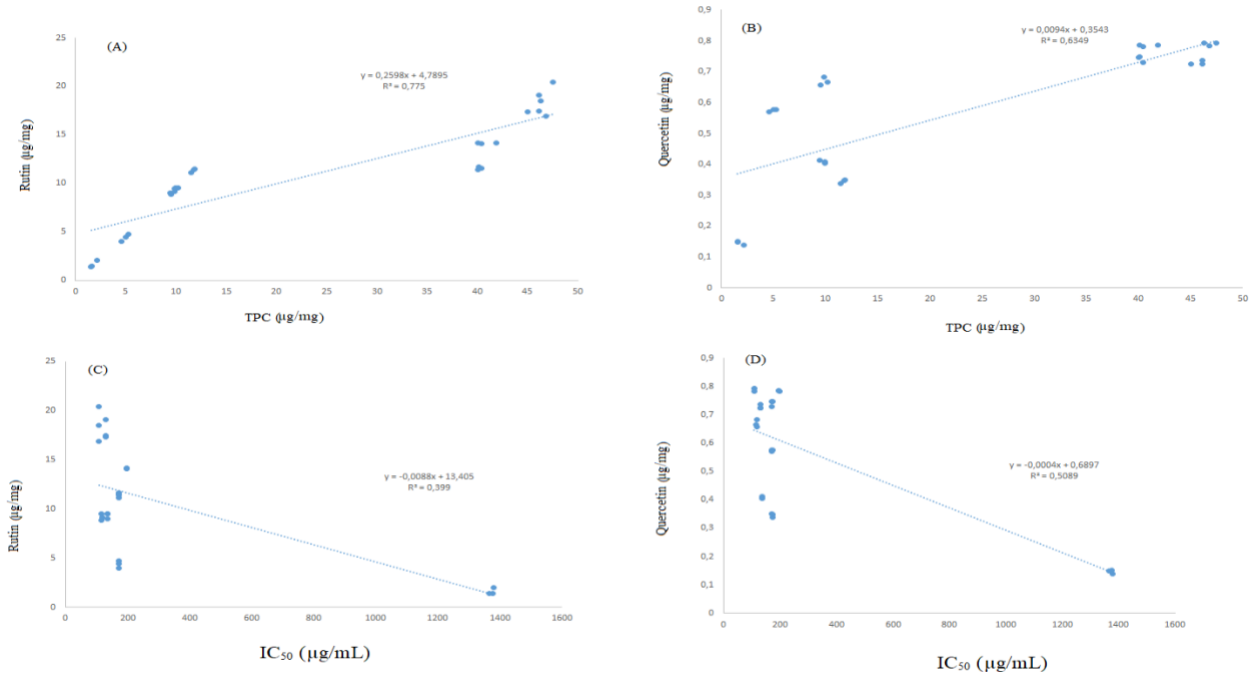

Figure 8. Correlation of the contribution of active compounds: A) Rutin Vs TPC; B) Quercetin vs TPC; C) Rutin Vs Inhibition activity of DPPH (IC_{50}); D) Quercetin Vs Inhibition activity of DPPH (IC_{50})

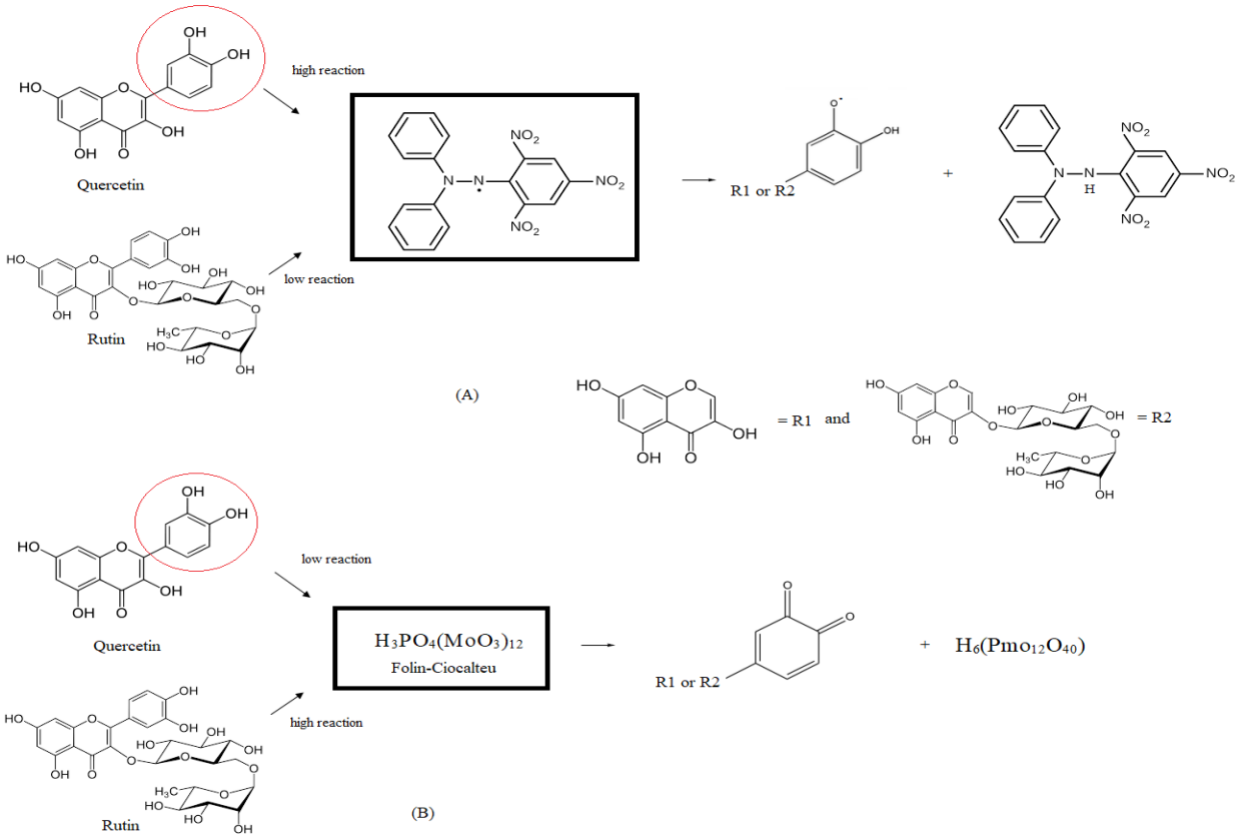

Figure 9. The oxidation reaction of DPPH radicals (A) and Folin Ciocalteu reagent (B)

4. Conclusions

This study showed that 60% ethanolic extract and 80% ethanolic extract of *C. caudatus* leaves had better antioxidant activity than other extracts. The optimum raw material to solvent ratio is 1:6 (b/v) containing rutin and quercetin concentrations around 18.59±1.77 µg/mg and 0.728±0.006 µg/mg, respectively. This study also concluded that quercetin is one of the active compounds from *C. caudatus* that plays a significant role in the DPPH radical scavenging, with better inhibition activity than rutin. Furthermore, TPC has a moderate correlation with DPPH inhibitory activity, as evidenced by Pearson's Correlation (r) < 0.6.

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