Original article

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Microscopic Characteristics, Total Phenolic Content and Antioxidant Activities of Root and Stem from *Sida acuta* Burm. F. extracts

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Abstract

Sida acuta (SA) belongs to the family Malvaceae. It is a medicinal plant that has long been used in Thai traditional and alternative medicine. It is used in the composition of various medicinal formulations such as anti-edema, analgesic and nourishing elements. The objectives for this study were to characterize microscopically, analyze the total phenolic content by Folin-Ciocalteu method and investigate antioxidant activities by DPPH and FRAP methods of roots and stems from SA extracts. SA were extracted using three methods as follows; maceration with 95% ethanol, maceration with 50% ethanol and decoction. The microscopical characteristics of SA powder have distinguishing features such as a large number of fibers and vascular bundles both in stems and roots. The most vascular bundle types are bordered pitted and reticulate vessels. The result of total phenolic content found that the roots and stems extract from SA are in the range of 16.97±0.42 to 86.19±1.00 mg GAE/g extract. The 95% ethanolic extract from roots of (SARE95) showed the highest total phenolic content of 86.19±1.00 mg GAE/g extract. The DPPH method showed that the roots had better antioxidant activity than the stems compared to extraction with the same solvent. It was determined that 50% ethanolic extract of the root (SARE50) had the best antioxidant activity, followed by 95% ethanolic extract of the root (SARE95) with EC_{50} values of 48.05±1.23 and 55.38±0.56 µg/mL, respectively. The 95% ethanolic extract of roots (SARE95) contained the highest FRAP and TEAC (137.07±2.73 mg Fe(II)/g extract and 57.77±1.31 mg Trolox/g extract). The roots and stems extracts demonstrated good antioxidant activities. Therefore, future investigations of the active substances and other biological activities are warranted.

Keywords: Antioxidant activity, Microscopic Characteristics, *Sida acuta*, Total phenolic content

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Introduction

Antioxidants had a growing interest owing to their protective roles in food and herbal against oxidative deterioration in the body and against oxidative stress. Screening of antioxidant properties of plants and active compounds requires appropriate methods. Many studies evaluating the antioxidant activity of various samples using different methods (Gulcin, 2020). Oxidative stress involves in many acute and chronic diseases including cancer, cardiovascular pathologies, rheumatoid arthritis and neurode-generative disorders such as Alzheimer's and Parkinson's diseases (Bouayed *et al*., 2007). Antioxidants are known to play an important role in protection against oxidative damage. Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity (Soobrattee *et al*., 2005).

Sida acuta (SA) belongs to the family Malvaceae. It is a medicinal plant that has long been used in Thai traditional and alternative medicine. It is used in the composition of various medicinal formulations such as anti-edema, analgesic and nourishing elements. Previous studies on biological activities of SA; petroleum ether, toluene, chloroform, acetone, ethyl acetate and hydroalcoholic extracts of whole plant of aerial part of SA showed the presence of alkaloids, carbohydrates, flavonoids, tannins, saponins, aminoacids, proteins and glycosides in various extracts. In vitro DPPH scavenging assay, toluene , chloroform and ethyl acetate extracts of SA showed significant IC_{50} values (78±2, 142.33±2.51 and 113±3.60 µg/mL), respectively (Thondawada *et al*., 2016). The preliminary photochemical screening of the aqueous leaf extract of S. acuta showed the presence of alkaloids, steriods, flavonoids, phenols, terpenoids, and cardiac glycosides (Senthilkumar *et al*., 2018). The chloroform and ethanolic extract of *S. acuta* showed the presence of carbohydrates, alkaloids, phytosterols, saponins and fixed oils (Palaksha & Ravishankar 2012). A gas chromatography-mass spectrometry (GC-MS) analysis of the SA leaves extract showed that 1,3-Propanediol, 2-(hydroxymethyl)-2-nitro (9.52%), 9,12,15-Octadecatrienoic acid, ethyl ester (Z,Z,Z) (7.68%), alpha, and 4- hydrocy-e-methylacetophenone (9.12%) were the most common bioactive compounds (Nalini *et al*., 2024). SA bark and roots are among the most effective parts of the SA for treating a variety of diseases. The root of SA is used extensively as an astringent, cooling agent, stomach problems, diaphoretic and antipyretic (Srinivasan & Murali 2022). Thus, this study aimed to characterize microscopically, analyze the total phenolic content by Folin-Ciocalteu method and investigate antioxidant activities by DPPH and FRAP methods of roots and stems from SA to confirm the properties in antioxidants.

Research Methodology

Microscopically study

Microscopical study analyzed the roots and stems of *Sida acuta* (SA). We harvested plant samples from the herb garden at Abhaibhubejhr College of Thai Traditional Medicine, Prachinburi in November until January. SA annually grows completely during this period. Plants specimens; no. BKF 228286, were identified by experts of Forest and Plant Conservation Research Office, Thailand. The stems and roots samples were dried, crushed, powdered and glided through a sieve size 180 µm. These powders were sealed in slides with distilled water and covered with cover glass. Microscopical characteristics were collect by light microscopy and connected with a digital camera (Bureau of Drug and Narcotic, 2017).

Preparation of plant extracts

The roots and stems of SA from the herb garden from Abhaibhubejhr College of Thai Traditional Medicine, Prachinburi were washed to remove extraneous contaminants, cut into small pieces and dried in a hot air oven- dried at 50°C and then ground into powder with an electric- grinder. The traditional medicines used are commonly made by boiling the plant material in water or soaking in ethanol. Therefore, SA was extracted using three methods as follows; maceration with 95% ethanol, maceration with 50% ethanol and decoction.

Maceration

In ethanol extraction 95% ethanol and 50% ethanol were used in the maceration. The roots and stems powders of SA were macerated at room temperature 3 times over 3 days and filtered through Whatman No. 1 paper. Then the extracts were combined and dried by rotary evaporator. Percentage yields of all extracts were calculated.

Decoction

The roots and stems powders of SA were boiled in distilled water of boiling point at temperature 100°C for 15 min, 3 times. The extracts were filtered through a Whatman No. 1 paper. Then the extracts were combined and dried by water bath at temperature 100°C and incubated by hot air oven at temperature 45°C until stable weight. Percentage yields of all extracts were calculated.

Determination of the extraction yield

The yields of the extracts based on dry weight were calculated from the following equation:

$$\% \text{ Yield} = \frac{\text{Weight of dry extract (g)}}{\text{Weight of the starting dry plant material (powder) (g)}} \times 100$$

The crude extracts were weighed and stored at freezer (-20 °C) until use.

Determination of total phenolic content

The total phenolic contents in the extracts were determined by using Folin-Ciocalteu's method (Folin & Ciocalteu, 1927). Gallic acid was used as a standard phenolic compound. It was dissolved in absolute ethanol to concentration of 1 mg/ ml. To prepare a standard calibration curve, a standard stock solution was diluted 7 concentrations in a serial dilution of 5, 10, 25, 50, 100, 250 and 500 µg/ml, respectively. For sample preparation 1 mg/ ml, the 95% ethanolic extracts were dissolved in absolute ethanol, the 50% ethanolic extracts were dissolved in 50% ethanol and water extracts were dissolved in distilled water. After that, 20 µl of the sample was added into 96 well-microplates and mixed thoroughly with 100 µl the Folin- Ciocalteu reagent and 80 µl sodium carbonate. The plate was incubated for 30 minutes at room temperature. The absorbance was measured at 765 nm using microplate reader spectrophotometer. The amount of total phenolic compounds in the extracts were determined as milligram gallic acid equivalent (mg of GAE/ g extract) using an equation obtained from the standard calibration graph.

***In vitro* assay for antioxidant activity; DPPH radical scavenging assay**

The antioxidant activity was determined using a DPPH assay, according to the modified method of Yamasaki *et al.*, 1994. A DPPH solution in absolute ethanol (6×10^{-5}M) was freshly prepared and kept at 4°C in a bottle protected from light before use. Butylated hydroxytoluene (BHT) was used as reference standard, prepared in absolute ethanol to 1 mg/ml concentration. For sample preparation 1 mg/ ml, the 95% ethanolic extracts were dissolved in absolute ethanol, the 50% ethanolic extracts were dissolved in 50% ethanol and water extracts were dissolved in distilled water. The samples were diluted to 1, 10, 50 and 100 µg/ ml, respectively. 100 µl of sample solution was added into 96 well- microplates and then added 100 µl of DPPH solution (6×10^{-5} M) in 96 well-microplates into each well. Incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 520 nm (Brand-Williams *et al.*, 1995). All tests were determined in triplicate. The effective concentration of sample required to scavenge DPPH radical by 50%: EC_{50}, was calculated by GraphPad Prism 8 for windows. The values were reported as mean±SEM of three replicate. The scavenging activity of the samples were calculated as a percent inhibition in the formula below:

$$\% \text{ Inhibition} = \frac{\text{Optical density (OD)}_{\text{control}} - \text{Optical density (OD)}_{\text{sample}} \times 100}{\text{Optical density (OD)}_{\text{control}}}$$

***In vitro* assay for antioxidant activity; Ferric ion reducing antioxidant power (FRAP) assay**

The FRAP assay is a method for measuring total reducing power of an electron-donating substance (Benzie & Strain, 1999). The FRAP working reagents were prepared as follows: (A) Acetate buffer 300 mM pH 3.6, (B) TPTZ (2, 4, 6-tripyridyl- s- trizine) 10 mM in 40 mM HCl and (C) Ferric chloride ($FeCl_3$) 20 mM solution. This solution was stored at room temperature. The fresh working FRAP reagent was prepared by mixing A, B and C in the ratio of 10:1:1 (A:B:C) at the time of use (Refreshing reagent). Finally, FRAP reagents were mixed

together. Trolox and Fe(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) were used as reference standard. Trolox was dissolved in absolute ethanol and diluted to 6 concentrations in a serial dilution of 5, 25, 50, 100, 200 and 300 µg/ml, respectively. Fe(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was dissolved in distilled water and diluted to 6 concentrations in a serial dilution of 5, 25, 50, 100, 200 and 300 µg/ml, respectively. For sample preparation 1 mg/ml, the 95% ethanolic extracts were dissolved in absolute ethanol, the 50% ethanolic extracts were dissolved in 50% ethanol and water extracts were dissolved in distilled water. 20 µl of the diluted sample solution was added into 96 well-microplates and then added 180 µl of FRAP reagent warmed at 37ºC for 4 minutes into each well. The absorbance at 593 nm was measured after incubation for 8 minutes at room temperature (Firuzi *et al*., 2005). The results were expressed as FRAP values and Trolox equivalent antioxidant capacity (TEAC). The FRAP values of the samples were determined from a calibration curve of ferrous sulfate solution. TEAC were determined from a calibration curve of Trolox.

Results & Discussion

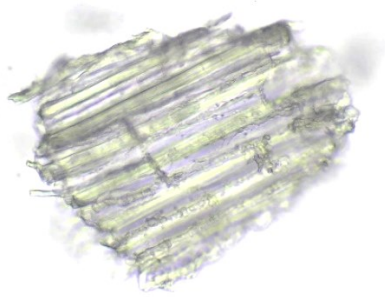
Characteristics of powder

(Mohideen *et al*., 2002; Nduche & Offor 2019).

Stem: Powder was light brown and spongy. The fibers are dense (1.1A) and scattered in all slides (1.1D). Calcium oxalate crystals are intracellular (1.1E) or next to vascular bundles (1.1G). We determined groups of vascular bundles such as bordered pitted vessels (1.1H) and scalariform vessels (1.1B). In the stem collenchyma cells (1.1C) and parenchyma cells were determined.

Root: Powder was light green and spongy, but lighter than the powder of stem. There are a significant number of white fibers. The root had trichomes present (1.2E) and groups of vascular bundles and fibers such as pitted vessels (1.2A), reticulately vessels (1.2B) group of fibers (1.2C) connected to vessels (1.2F).

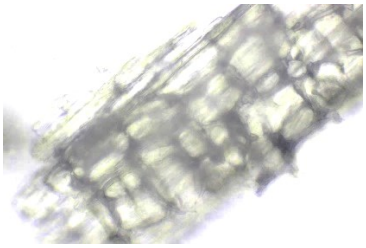
The microscopical characteristics of SA powder have distinguishing feature are a large numbers of fibers and vascular bundles both in stems and roots. The majority of vascular bundles types are bordered pitted and reticulate vessels. These findings can be used to standardize the herbal characteristics.



1.1A Group of fibers (40x)



1.1B Scalariform vessels (40x)



1.1C Collenchyma cells (40x)

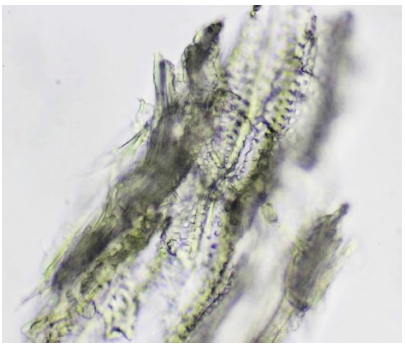


1.1D Fragment of fibers (10x)

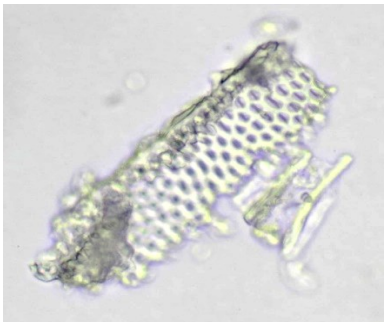




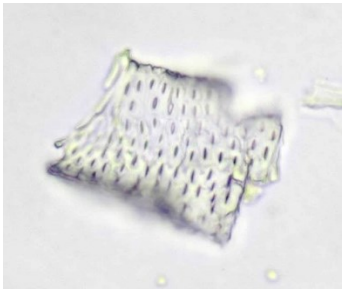
1.1E Group of fibers with part of calcium oxalate (40x)



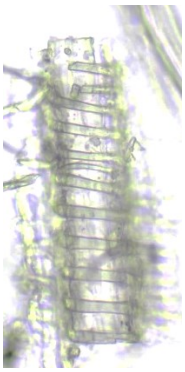

1.1G Group of fibers with bordered pitted vessels (10x)

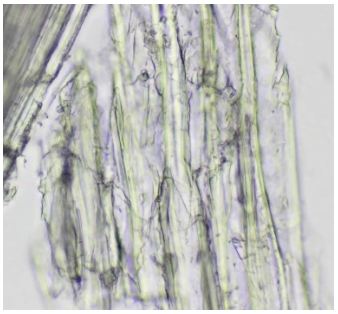


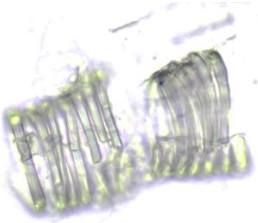
1.1H Bordered pitted vessels (40x)



1.2A fragment of pitted vessels (40x)



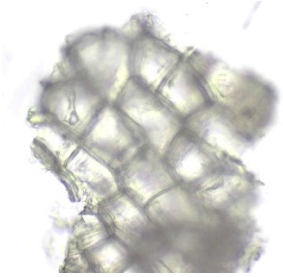


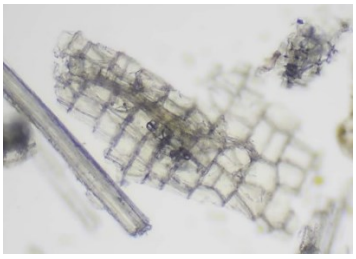


1.2B fragment of reticulately vessels (40x)

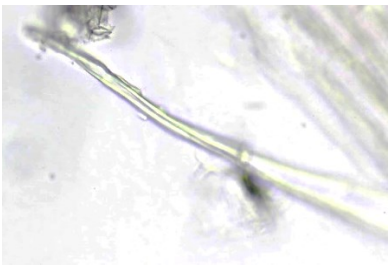


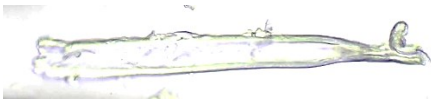
1.2C group of fibers (10x)



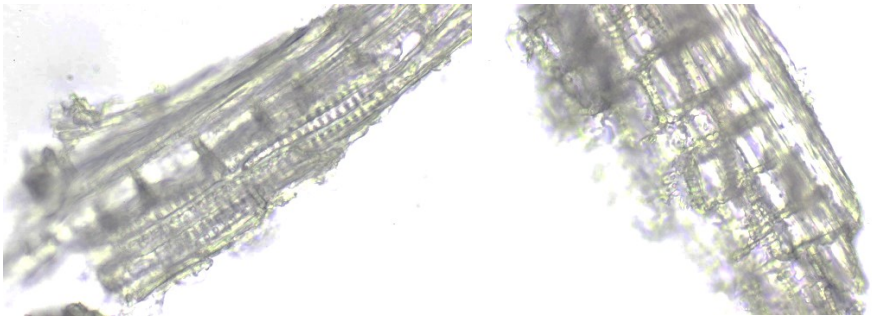


1.2D Parenchyma cells (40x)





1.2E simple trichomes (10x)



1.2F Group of fibers with bordered pitted vessels (10x)

Determination of the extraction yield

The percentage of yield of roots and stems from SA are shown in **Table 1**. The percentage of yield of roots extracts in range from 2.81% to 1.03% and the percentage of yield of stems extracts in range from 3.83% to 2.38%. The 50% ethanolic extract from stems of SASE50 showed the highest percentage of yield.

Table 1. Percent yield of crude extracts of *Sida acuta* by different extraction methods

Sida acuta	**Code**	**% Yield**		
		95% ethanol (E95)	**50% ethanol (E50)**	**water (W)**
Root	SAR	1.03	1.61	2.81
Stem	SAS	2.38	3.83	3.44

Determination of total phenolic content

The absorbance at 765 nm was plotted with the 7 concentrations of standard Gallic acid solution showed in **Figure 1**. The representative regression coefficient (R^2) was 0.9998 and the linear regression equation was y = 0.0055x + 0.0603. Total phenolic content was reported as mg GAE/g extract showed in **Table 2**. The results of this study showed that the roots and stems extract from SA was in the range from 16.97±0.42 to 86.19±1.00 mg GAE/g extract. The 95% ethanolic extract from roots of SARE95 showed the highest total phenolic content of 86.19±1.00 mg GAE/g extract, followed by the 95% ethanolic extract from stems of SASE95, the 50% ethanolic extract from roots of SARE50, the 50% ethanolic extract from stems of SASE50, the water extract from roots of SARW and the water extract from stems of SASW showed the total phenolic contents of 72.31±1.04, 36.19±0.32, 25.10±0.12, 19.16±0.16 and 16.97±0.42 mg GAE/g extract, respectively. Studies that investigated the impact of temperature on polyphenolic yields have provided insight into the behaviors of polyphenols. Generally, for traditional extractions, the total phenolic content is highest at an extraction temperature of 60-80 °C at temperatures above 80°C, the polyphenolic yield decreases. For this reason, polyphenols are heat-labile compounds. (Antony & Farid, 2022). Which is consistent with the results of this research, the total phenolic content from each extract were different.

The previous study found that the ethanolic extract from leaves of SA showed the total phenol, flavonoid, tannin and alkaloid content was found to be 31 ± 0.15 (mg/g gallic acid equivalent), 40 ± 0.25 (mg/g quercetin equivalent), 50 ± 0.2 (mg/g catechin equivalent) and 0.025 ± 0.1 (mg/10 mg of plant extract) respectively. The results indicated a higher concentration of polyphenols, whereas, alkaloids in a minimal quantity (Muneeswari *et al*., 2019). Methanolic extract from roots of SA showed the total phenolic content 1.24±0.06 mg caffeic acid equivalent/g and 1.45±0.07 mg Tannic acid equivalent/g (Subramanya *et al*., 2015).

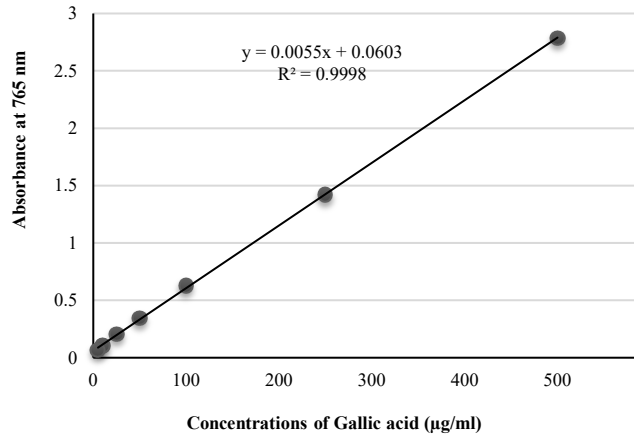


Figure 1 Calibration curve between Gallic acid standards solution (µg/ml) and absorbance values at 765 nm (n=3)

Table 2. Total phenolic content of roots and stems extracts from *Sida acuta* (Mean±SEM, n=3)

Plant species	Extracts	Code	Total phenolic content (mg GAE/g extract)
Root	95%EtOH	SARE95	86.19±1.00
	50%EtOH	SARE50	36.19±0.32
	Water	SARW	19.16±0.16
Stem	95%EtOH	SASE95	72.31±1.04
	50%EtOH	SASE50	25.10±0.12
	Water	SASW	16.97±0.42

In vitro assay for antioxidant activity: DPPH radical scavenging activity

The results of antioxidant activity using DPPH assay of roots and stems extracts from SA is shown in **Table 3**. The roots and stems extract from SA demonstrated EC_{50} values in the range from 48.05±1.23 to 84.62±0.82 µg/ml. The 50% ethanolic extract from roots of SARE50 showed the highest antioxidant activity with EC_{50} value of 48.05±1.23 µg/ml. BHT as a positive control (EC_{50} = 14.13±0. 21 µg/ ml) revealed higher antioxidant activity than the roots and stems extracts from SA.

The previous study found that the petroleum ether extract from leaves of SA exhibited the potential free radical scavenging activity (antioxidant activity) having IC_{50} value of 98. 93 µg/ ml (Bahar *et al*., 2013). The petroleum ether, toluene, chloroform, acetone, ethyl acetate and hydroalcoholic extracts of whole plant of SA showed antioxidant activity using DPPH assay with IC_{50} values of 321 ± 4.00, 78 ± 2.00, 142.33 ± 2.51, 194.33 ± 4.04, 113 ± 3.60 and 402 ± 2.51 µg/ml, respectively (Thondawada *et al*., 2016). Compared to previous studies, this study showed that antioxidant activity using DPPH assay of roots and stems extracts from SA were better tendency than other solvents but should be use exactly the same methodology.

Table 3. The percent inhibition of roots and stems extracts from *Sida acuta* at concentration 100, 50, 10 and 1 µg/ml and EC_{50}±SEM (µg/ml) using DPPH radical scavenging activity (n=3)

Sida acuta	Extracts	Code	%Inhibition at concentration±SEM (µg/ml)				EC_{50} (µg/ml)
			100	50	10	1	
Root	95% EtOH	SA RE 95	65.85 ± 0.80	44.58 ± 0.56	3.83 ± 0.69	0.18 ± 0.09	55.38 ± 0.56
	50% EtOH	SA RE 50	94.35 ± 0.72	51.70 ± 1.15	15.63 ± 1.78	7.26 ± 2.04	48.05 ± 1.23
	Water	SA RW	90.01 ± 1.04	41.23 ± 0.95	4.28 ± 0.83	1.52 ± 0.32	57.65 ± 0.79
Stem	95% EtOH	SA SE 95	62.00 ± 0.80	38.78 ± 1.13	6.88 ± 0.91	3.12 ± 0.65	65.51 ± 0.42
	50% EtOH	SA SE 50	52.42 ± 0.95	37.96 ± 0.89	7.43 ± 1.00	3.16 ± 1.13	74.44 ± 1.12
	Water	SA SW	56.92 ± 0.17	31.26 ± 0.78	6.01 ± 2.23	1.06 ± 2.66	84.62 ± 0.82
Positive control		BHT	83.25 ± 0.26	77.01 ± 0.51	38.22 ± 0.15	4.14 ± 1.33	14.13 ± 0.21

In vitro assay for antioxidant activity: Ferric ion reducing antioxidant power (FRAP) assay

The FRAP values was calculated from the graph standard of ferrous sulfate ($FeSO_4 \cdot 7H_2O$). A linear correlation was observed between the absorbance at 593 nm and 6 concentrations a series of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) solution. The representative regression coefficient (R^2) was 0.9999 and the linear regression equation was y = 0. 0044x+ 0. 1829 **Figure 2**. The results were showed in **Table 4**. The roots and stems extracts from SA showed FRAP values in the range from 137.07±2.73 to 66.29±2.11 mg Fe(II)/g extract. Comparison, FRAP values from roots better than stems in the same solvent. The 95% ethanolic extract from roots of SARE95 showed high

antioxidant activity with FRAP value of 137.07±2.73 mg Fe(II)/g extract. FRAP value of BHT as 273.96±0.40 mg Fe(II)/g extract. The Trolox equivalent antioxidant capacity (TEAC) values were calculated from the graph standard of Trolox. A linear correlation was observed between the absorbance at 593 nm and 6 concentrations a series of Trolox solution. The representative regression coefficient (R^2) was 0.9998 and the linear regression equation was y = 0.0092x+0.2545 **Figure 3**. The results were showed in **Table 5**. The roots and stems extract from SA showed TEAC values in the range from 57.77±1.31 to 24.26±1.01 mg Trolox/g extract. Comparison, FRAP values from roots better than stems in the same solvent. The 95% ethanolic extract from roots of SARE95 showed high antioxidant activity with TEAC value of 57.77±1.31 mg Trolox/g extract. TEAC value of BHT as 123.24±0.19 mg Trolox/g extract. The previous study found that the FRAP assay was demonstrate the reducing power of the ethanolic extract from leaves of SA as well as the standard which increases with the increase in amount of sample and standard concentrations. This is reflective of a better radical scavenging tendency of the SA leaf extract than the standard ascorbic acid (Muneeswari *et al.,* 2019). The results from SA by FRAP assay corresponded to total phenolic content. The antioxidant results (DPPH and FRAP assay) correspond to total phenolic content from stems extract. The roots extract SARE50 showed the highest antioxidant activity but 95% ethanolic extract from roots of SARE95 showed the highest total phenolic content, FRAP value and TEAC value; It is possible that; the compounds were potency antioxidant activity non phenolic and active ingredient were extracted with water and ethanol solvent in a proportion of 50% ethanol. In principle "Like dissolves like" means if any substance is polar then polar and ionic substances will be dissolved in it. 50% ethanol more polar than 95% ethanol.

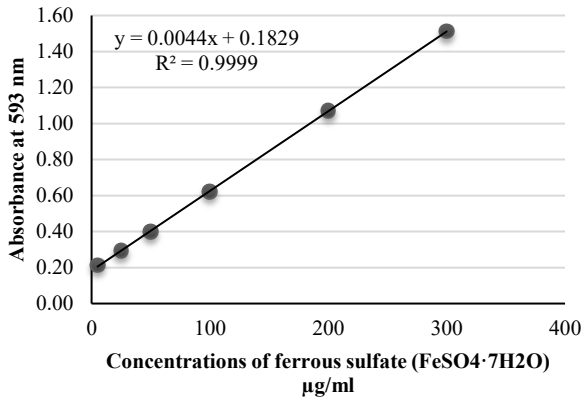


Figure 2 Calibration curve between the absorbance at 593 nm by FRAP assay as 6 concentrations of Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) standards solution (n=3)

Table 4. Antioxidant activity of roots and stems extracts from *Sida acuta* expressed as the FRAP values±SEM measuring by FRAP assay (n=3)

Sida acuta	Extracts	Code	FRAP values (mg Fe(II)/g extract)
Root	95%EtOH	SARE95	137.07±2.73
	50%EtOH	SARE50	95.10±0.53
	Water	SARW	87.22±1.38
Stem	95%EtOH	SASE95	105.10±1.33
	50%EtOH	SASE50	89.49±1.55
	Water	SASW	66.99±2.11
Positive control		BHT	273.96±0.40

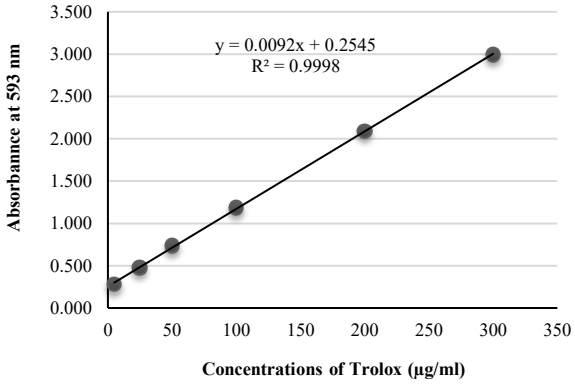


Figure 3 Calibration curve between the absorbance at 593 nm by FRAP assay as 6 concentrations of Trolox standards solution (n=3)

Table 5. Antioxidant activity of roots and stems extracts from *Sida acuta* expressed as the Trolox equivalent antioxidant capacity (TEAC) values±SEM measuring by FRAP assay (n=3)

Sida acuta	Extracts	Code	TEAC values (mg Trolox/g extract)
Root	95%EtOH	SARE95	57.77±1.31
	50%EtOH	SARE50	37.70±0.25
	Water	SARW	33.93±0.66
Stem	95%EtOH	SASE95	42.48±0.63
	50%EtOH	SASE50	35.02±0.74
	Water	SASW	24.26±1.01
Positive control		BHT	123.24±0.19

Conclusion

The results shown in **Table 6**, total phenolic content and antioxidant activities using DPPH assay, FRAP and TEAC assay were found that the roots extracts from SA showed activity better than stems extracts in the same solvent. Therefore, these results are good preliminary study points for used the SA extract to be investigations of the active substances and other biological activities are warranted.

Table 6. Summary of antioxidant activities and determination total phenolic content of roots and stems extracts from *Sida acuta* (n=3)

Code	Total phenolic content (mg GAE/ g extract)	DPPH assay EC_{50} (µg/ml)	FRAP assay	
			FRAP values (mg Fe(II)/g extract)	TEAC values (mg Trolox/g extract)
SARE95	86.19±1.00	55.38±0.56	137.07±2.73	57.77±1.31
SARE50	36.19±0.32	48.05±1.23	95.10±0.53	37.70±0.25
SARW	19.16±0.16	57.65±0.79	87.22±1.38	33.93±0.66
SASE95	72.31±1.04	65.51±0.42	105.10±1.33	42.48±0.63
SASE50	25.10±0.12	74.44±1.12	89.49±1.55	35.02±0.74
SASW	16.97±0.42	84.62±0.82	66.99±2.11	24.26±1.01
BHT	-	14.13±0.21	273.96±0.40	123.24±0.19

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