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Original Research Article

Phytochemical Screening, Total Phenolic, Reducing Sugar Contents, and Antioxidant Activities of *Gelidium spinosum* (S.G. Gmelin) P.C. Silva

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ABSTRACT

Gelidium spinosum is edible red seaweed with high economic values and potential pharmacological activities. This research aimed to evaluate phytochemicals, total phenolic content, reducing sugar content, and antioxidant properties of *Gelidium spinosum* methanol-aqueous extracts. Maceration with different solvent ratios of methanol-water was employed to afford various crude extracts. The standard procedures of preliminary phytoconstituents determination were employed to screen the presence of various phytochemicals. Phenolic and reducing sugar contents were determined using Folin-Cioucalteu and 3,5-dinitro salicylic acid methods. The antioxidant activities of seaweed extracts were determined through DPPH and reducing power assays. The 100% methanol extract of *G. spinosum* was found to be rich in alkaloids, flavonoids, glycosides, polyphenols, proteins, reducing sugar, saponins, steroids and tannins. The aqueous extract of *G. spinosum* contained flavonoids, glycosides, polyphenols, reducing sugars, saponins and tannins at a moderate level. The total phenolic content range was 6.43 to 49.78 mg EGA/g extract. The highest reducing sugar content was shown by 100% methanol extract (1278.20 ± 21.25 mg GE/g extract). The highest antioxidant activities were found in 100% methanol extract for reducing power assay, and 75% methanol extract of *G. spinosum* for DPPH method. There was a positive correlation between reducing sugar, total phenolic contents and antioxidant activities. Results further confirmed the potential use of red seaweed in various ailments, however, should further be confirmed through more appropriate similar studies.

Keywords: *Gelidium spinosum*, red seaweed, phytochemicals, phenolic content, reducing sugar content, DPPH, reducing power

Introduction

Red seaweed is a commodity source of food ingredients originating from the ocean. Seaweed is included in the plant division *Thallophyta*. Red seaweed contains numerous phytochemicals with interesting capabilities and a wide range of uses.[1-5] Red seaweed is traditionally used as animal feed, fertility, food, and a source of hydrocolloids.[6] In traditional medicine, seaweed is used to prevent influenza, colds, tuberculosis, arthritis, rheumatism, and other diseases.[7] Meanwhile, red seaweed is widely used in a wide range of sectors, including the cosmeceutical, culinary, nutraceutical, and pharmaceutical ones.[8-9]

Gelidium spinosum is commonly known as edible red seaweed with high economic values. The classification of the plant includes *Rhodophyta* division, *Eurhodophytina* sub-division, *Florideophyceae* class, *Rhodymeniophycidae* sub-class, *Gelidiales* order, *Gelidiaceae* familia, *Gelidium* genus and G*elidium spinosum* (S.G. Gmelin) P.C. Silva as a species.

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G. spinosum can be found in areas along the Indonesia coast. Red seaweed from the genus is used in the culinary and pharmaceutical sectors as a source of agar.[10] Agar is used as a thickener and stabilizer in the confectionery industry and food industry to create food products, such as jelly candies. Agar is frequently used as a substitute for gelatin in diets for losing weight.[6] Consuming a healthy diet lowers the chance of developing chronic disorders includes atherosclerotic coronary artery,[11] type-2 diabetes,[12] and cardiovascular problems (hypertension, dyslipidemia, obesity, and metabolic syndrome).[13] Some conditions develop as a result of oxidative stress, which is caused on by biological systems producing too many reactive oxygen substances or ROS. The integrity of macromolecules including DNA, lipids, and proteins can be impacted by increased ROS.[14] These impacts can be controlled with a balance between ROS production and antioxidants. Free radicals can be eliminated and captured by antioxidant compounds.[15] Internal antioxidants already exist in the body, while external antioxidants can be obtained from natural sources.[16] Antioxidants mostly can be found in plants in the form of various chemical constituents, such as flavonoids, alkaloids, phenolic compounds, terpenoids, and vitamins.[17-20]

Several previous studies have reported the phytoconstituents of the *Gelidium* genus. Mohy El-Din and Al-Agawany highlighted that the red algae belongs to this genus obtained from the north coast of Alexandria (Egypt), contain phenolic compounds (phenols and flavonoids), tannins, carbohydrates and sulfates.[21] Additionally, they revealed the presence of alkaloids, saponins, anthocyanin, sugar, and C-heterosids as important constituents.[22] *G. spinosum* has been reported to display an α-amylase inhibitory activity supporting its applicability to diabetic patients as a supplement.[23] To date, no study has investigated methanol-aqueous extract from *G. spinosum* and its activities. Therefore, the

current study further focused on evaluating the phytochemicals analysis of *G. spinosum* methanol-aqueous extracts and their antioxidant activities. The extracts were obtained with different ratios of methanol-water as solvent extraction. Moreover, this study determined the total phenolic and reducing sugar contents, DPPH and the reducing power properties of methanol and aqueous extracts of *G. spinosum* for the first time. In addition, this study also investigated the correlation between (i) reducing sugar and phenolic contents, (ii) reducing sugar content and reducing power, and (iii) total phenolic content and DPPH activities.

Materials and Methods

Chemical materials

Ascorbic acid standard, $CuSO_4$, CH_3COOH, $CHCl_3$, Dragrondroff's reagent, HCl, H_2SO_4, KH_2PO_4, $FeCl_3.6H_2O$, Folin-Cioucalteu's phenol reagent, Na_2CO_3, NaOH, methanol, Mayer's reagent, magnesium metal, Molisch's reagent, potassium ferricyanide, and trichloroacetic acid were bought from Merck (Darmstadt, Germany). Compounds of 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3,4-dinitro salicylicic acid (DNSA), gallic acid and galactose were obtained from Sigma-Aldrich (St. Louis Missouri, USA). Potassium sodium tartrate ($KOCO[CHOH]_2COONa.4H_2O$) was procured from J.T. Baker. Unless otherwise noted, all reagents were of an analytical reagent grade.

Plant materials

Red seaweed (*G. spinosum*) was harvested in January 2020 from Drini, Gunungkidul, Yogyakarta, Indonesia coastline. The sample was authenticated in the Laboratory of Plant Systematics, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia (Document no. 0147/S.Tb./X/2022).

Preparation of extracts

The fresh red seaweed was initially cleaned with water and allowed to air dry. The dried red seaweed was pulverized to obtain its powder form. The 40-mash was used to sift the seaweed powder. Twenty grams of dried red seaweed powder was extracted using various ratios of methanol-water (100:0, 75:25, 50:50, 25:75, and water) in a ratio of 1:8. The extraction was carried out in a waterbath shaker for 24 hours. In a Büchner vacuum, the extracts were separated utilizing filter paper (Whatman number 1). The residues were once again thoroughly mixed with varied methanol-water ratios. A rotary vacuum evaporator was used to concentrate the extracts at 60-65°C. The extracts were then freeze dried. Finally, the freeze dried seaweed extracts were carefully put in a deep freezer at -20°C until further analysis.[24] Extraction of each variation was created in triplicates.

Phytochemicals screening

Preliminary qualitative phytoconstituents assessment of *G. spinosum* extract was conducted by chemical reactions as described by the following methods.[10,25] This analysis was subjected to 100% methanol extract and aqueous extracts of *G. spinosum*. The analyses carried out were for the present of alkaloids (Mayer's and Dragrondroff's reagents), flavonoids (magnesium metal + conc. HCl), glycosides (CH_3COOH + 5% $FeCl_3$ + conc. H_2SO_4), polyphenols (extract in water + 5% $FeCl_3$), protein (1% NaOH + 1% $CuSO_4$), reducing sugar (Molisch's reagent), saponins (present of effervescent), steroids ($CHCl_3$ + conc. H_2SO_4), and tannins (extract in water + 5% $FeCl_3$).

Assessing of phenolic content

The total phenolic content (TPC) was determined through Folin-Cioucalteu's phenol assay,[26] with minor modifications. This analysis was subjected to all extracts prepared in this study. Mixture reaction was composed of seaweed extract (0.5 mL), Folin-Cioucalteu's phenol solution (0.5 mL) (1:5) and distilled water (0.5 mL). The mixtures were neutralized with 1.0 mL of 5 % Na_2CO_3. The mixture reactions were left at 30°C for 2 hours in the darkness. The absorbance was measured spectrophotometrically at 765 nm. The total phenolic content was represented as the mg equivalent of gallic acid (EGA)/g extract (10-70 µg/mL). The total phenolic content was estimated according to Equation 1 given below.

$$\text{Content of compound } \left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{X } \left(\frac{mg}{mL}\right) \text{ x Initial volume (mL)}}{\text{Weight of the sample (g)}} \quad (1)$$

Assessing of reducing sugar content

The reducing sugar content (RSC) was quantified through a method of 3,5-dinitro salicylic acid (DNSA), with a small adjustment. The measurement was conducted in accordance with the standard literature.[33] Solution of DNSA was generated by mixing 80 mL of NaOH (0.5 N), DNSA (0.25 g), and 7.5 g of potassium sodium tartrate at 45°C. The solution was set at 30°C and then added distilled water up to 100.0 mL. Each reaction's combination contained 0.5 mL of DNSA reagent and 0.5 mL of *G. spinosum* extract (1.0 mg/mL). The solution was then heated for 5 minutes at 95°C. The absorbance was estimated spectrophotometrically at λ 540 nm. The reducing sugar content was represented as mg galactose equivalent (GE)/g extract (0.2 to 1.3 mg/mL). Amount of RSC was calculated according to Equation 1.

In-vitro antioxidant evaluations

Reducing power activity

The reducing power (RP) assay was carried out following the previous study by the researcher,[22] with minor variations. The solution was composed of each different seaweed extract (1.0 mL in 75% methanol), 2.0 mL of $K_3[Fe(CN)_6]$ (1%) and 2.0 mL of phosphate buffer (0.2 M, pH 6.6). The reaction medium was heated at 50°C for 30 minutes. The solutions were added 2.0 mL trichloroacetic acid (10%) and shaken for 10 minutes at 3000 rpm. The supernatant was pipetted at 2.0 mL, then added with 0.5 mL of $FeCl_3$ (0.1%) and 2.0 mL of distilled water. A 1.0 mL of ascorbic acid (final concentration of 0.64–2.125 µg/mL) and 75% methanol (1.0 mL) were added to the mixture as the positive control and negative control, respectively, instead of sample (seaweed extracts). Absorbance was determined spectrophotometrically at 700 nm. 75% methanol was used as a blank. All the tests were replicated three times. The reducing power assay was quantified with $EC_{0.5}$ value. $EC_{0.5}$ is constituting a concentration at which absorbance was 0.5.

DPPH activity

The DPPH method was conducted by following the guidelines described in the literature[27] with slight adjustments. A 1.0 mL of DPPH 0.2 mM was added to 1.0 mL of extracts at different percentages as a reaction solution. The solution was kept for 30 minutes at 30°C in darkness. Methanol (1.0 mL) was used as negative control. A UV-Vis spectrophotometer was used to record the absorbance at 517 nm. Methanol with each different concentration of seaweed extract was used as a blank. The test was replicated three times. As a standard drug, ascorbic acid was used in concentration of 4.5 µg/mL. % inhibition was used as the parameter of DPPH activity. The % inhibition of DPPH was calculated using a formula as stated in Equation 2. The equation consists of A = negative control absorbance and B = seaweed extract absorbance.

$$\text{Inhibition (\%)} = \text{A} - \frac{\text{B}}{\text{A}} \text{ X } 100 \quad (2)$$

Data analysis

The experiments were conducted as triplicates. Data were displayed as an average ± standard deviations (SD). The correlations between RSC, TPC, and antioxidant capacities were calculated using Pearson's correlation coefficient (R) and coefficient of determination (R^2). The Statistical Package for Social Sciences (SPSS) 16.0 for Windows was used to analyse the data. These analyses included the Kolmogorov-Smirnov test to explore the normality, as well as the Levene test to know the homogeneity. The analysis was continued with one-way ANOVA and post hoc Tukey. The result was considered significant at p value < 0.05.

Results and Discussion

The yield of the crude G. spinosum extract

The seaweed, *G. spinosum*, plant is presented in Figure 1. Several variables, including solvent, duration, techniques, and temperature of extraction, have an impact on efficiency and yield extraction.[28-29] This recent study focuses on optimizing the extraction yield by using various

solvents ratios of methanol-water. *G. spinosum* was extracted using the maceration method, and different solvents ratios of methanol-water (100:0, 100:25, 50:50, 25:75, and water). Therefore, results of extracts presented namely 100% methanol extract, 75% methanol extract, 50% methanol extract, 25% methanol extract and aqueous extract of *G. spinosum*. Afterward, the obtained extract was dried using freeze-drying technique. Yield and extracts of this research are presented in Table 1. Result of extraction as stated in Table 1 showed that the resultant extract from various solvent ratios produced in different colors, from green to brownish. Previous research reported that the extraction of *Gelidium* sp. with water as a solvent had resulted in a 2.52% of yield[30] with the orange dry extract. This result describes that the extraction using more methanol, resulted in a slightly darker color of the obtained extract than the aqueous one.

Figure 1: Red seaweed (*Gelidium spinosum*)

Table 1: The obtained of *G. spinosum* extractions

***G spinosum* extract**	**Yield (%)**	**Color of extract**
100% Methanol extract	1.92 ± 0.01[a]	
75% Methanol extract	2.42 ± 0.05[b]	
50% Methanol extract	3.44 ± 0.22[c]	
25% Methanol extract	4.09 ± 0.23[d]	
Aqueous extract	5.98 ± 0.33[e]	

Different superscript letters in the same column show a significant difference ($p < 0.05$). Data are presented as mean ± SD (n = 3)

Phytochemicals screening

The phytochemicals screening was performed to determine the chemical constituents of the methanol-water extracts of *G. spinosum*. The representative extracts including 100% methanol and aqueous extracts of *G. spinosum* were selected for this assay. The results of phytochemicals screening are presented in Table 2. The result showed that the 100% methanol of *G. spinosum* is rich in alkaloids, flavonoids, glycosides, polyphenols, proteins, reducing sugar, saponins, steroids and tannins. However, the presence of flavonoids, glycosides, polyphenols, reducing sugars, saponins, and tannins at a moderate level of traces were observed in the aqueous extract of *G. spinosum*.

Total phenolic content

The total phenolic contents of the methanol and aqueous extracts of *G. spinosum* are displayed in Table 3. The total phenolic contents' determination of methanol and aqueous extracts of *G. spinosum* was conducted through Folin-Ciocalteau assay. The basic principle mechanism of this assay suggests an oxidation-reduction reaction by oxidizing the phenolic group and reducing molybdenum ion. The Folin-Ciocalteau phenol solution is composed of a mixture of phosphotungstate phosphomolybdate and heteropoly acids. The molybdenum-tungsten ($WO_4MO_4 = Mo^{+6}$) in this reagent is in the +6 oxidation state, which is yellow. Molybdenum-tungsten blue ($MoW_{11}O_{40} = Mo^{+4}$) is generated during a reaction by a hydroxyl group of phenolic compounds, and the average oxidation state of molybdenum is between 4 (Mo^{4+}) and 5 (Mo^{5+}). At acidic pH, the oxidation-reduction reaction proceeds slowly, whereas at basic pH, it proceeds quickly. Therefore, carbonate sodium was used to maintain the basic condition. The product's maximum reaction intensity is at a wavelength of 765 nm.[31] The total phenolic content was counted from the linear regression ($y = 0.0123x + 0.133$, $R^2 = 0.9818$) of standard gallic acid and stated as mg EGA/g extract). The total phenolic content from the methanol and aqueous extracts of *G. spinosum* ranged between 6.43 and 49.78 mg EGA/g extract. Similarly, 100% methanol extract had the highest total phenolic content, followed by 75%, 50%, 25% and aqueous extracts of *G. spinosum* ($p < 0.05$).

Reducing sugar content

A 3,5-dinitro salicylic acid assay was used to measure the reducing sugar content of methanol and aqueous extracts of *G. spinosum*.[14] Galactose is the major sugar content in seaweed plants. Therefore, in the determination of reducing sugar content, the same compound was used as a standard curve. The reducing sugar content of extracts was estimated from the linear regression of standard galactose ($y = 0.5785x + 0.1053$, $R^2 = 0.9962$). Reducing sugar content was stated in a value of mg galactose equivalent (mg GE)/g extract. The reducing sugar content of the extracts is summarized in Table 4. The highest reducing sugar content was found in the 100% methanol extract of *G. spinosum* (1278.20 ± 21.25 mg GE/g extract) with p value < 0.05.

Antioxidant activities evaluation

The study evaluated antioxidant activities, including the reducing power and DPPH assays of methanol and aqueous extracts of *G. spinosum*. Results of this evaluation are presented in Table 5. According to the reports, reducing power is a fundamental aspect of antioxidant activity. In this approach, ferricyanide complex (Fe^{3+}) is reduced to ferrous (Fe^{2+}) due to the presence of reductants in the antioxidant samples.[22] In this study, ascorbic acid was used as a standard drug. Antioxidant capacity was stated in $EC_{0.5}$ value. $EC_{0.5}$ is defined as a concentration of the extract, which can reduce oxidizing reagents with an absorbance of 0.5. The smaller the $EC_{0.5}$ value, the stronger the potential of the extract as an antioxidant. The $EC_{0.5}$ of the different extracts were obtained ranging from 26.85 ± 1.27 to 219.71 ± 3.90 µg/mL. Meanwhile, the $EC_{0.5}$ of ascorbic acid was found to be 1.70 ± 0.01 µg/mL. The 100% methanol extract of *G. spinosum* revealed the highest potential as an antioxidant ($p < 0.05$) for reducing power activity. Meanwhile, the 75% methanol extract of *G. spinosum* was found to be the most potential element ($p < 0.05$) for DPPH scavenging. However, the potency of these extracts were weaker when compared to ascorbic acid.

Furthermore, free radical scavenging activity of the *G. spinosum* extracts was determined through DPPH assay in a concentration of 0.2 mM. The results were stated as a % inhibition to DPPH as displayed in Table 5. The % inhibition of the extracts ranged from 76.95 ± 0.62% to 7.68 ± 0.61%. The order of DPPH scavenging activity against the extract was found to be in the decreasing order i.e., 100%, 75%, 50%, 25% methanol and aqueous extracts of *G. spinosum*.

Table 2: Phytochemical analysis of *G. spinosum* methanol and aqueous extracts

Tests	*G. spinosum* extracts	
	100% Methanol extract	Aqueous extract
Alkaloids	+	–
Flavonoids	+	+
Glycosides	+	+
Polyphenols	+	+
Protein	+	–
Reducing sugars	+	+
Saponins	+	+
Steroids	+	–
Tannins	+	+

– Absent, + Presence

Table 3: TPC of *G. spinosum* methanol and aqueous extracts

G. spinosum extracts	TPC (mg EGA/g extract)
100% Methanol extract	49.78 ± 1.56[a]
75% Methanol extract	39.05 ± 3.08[b]
50% Methanol extract	27.76 ± 0.37[c]
25% Methanol extract	13.48 ± 0.45[d]
Aqueous extract	6.43 ± 0.26[e]

Different superscript letters in the same column show a significant difference ($p < 0.05$). Data are displayed as mean ± SD (n = 3)

Table 4: RSC of *G. spinosum* methanol and aqueous extracts

G. spinosum extracts	RSC (mg GE/g extract)
100% Methanol extract	1278.20 ± 21.25[a]
75% Methanol extract	1014.83 ± 14.91[b]
50% Methanol extract	648.99 ± 24.02[c]
25% Methanol extract	376.07 ± 5.49[d]
Aqueous extract	321.99 ± 9.52[e]

Different superscript letters in the same column show a significant difference ($p < 0.05$). Data are served as mean ± SD (n = 3)

Table 5: Antioxidant activities of *G. spinosum* methanol and aqueous extracts

G. spinosum extracts	RP Assay $EC_{0.5}$ ± SD (µg/mL)	DPPH (% inhibition)
100% Methanol extract	26.85 ± 1.27[a]	76.95 ± 0. 62[a]
75% Methanol extract	31.53 ± 0.73[b]	79.82 ± 0.75[b]
50% Methanol extract	61.89 ± 1.16[c]	43.20 ± 2.02[c]
25% Methanol extract	103.33 ± 1.48[d]	19.71 ± 0.86[d]
Aqueous extract	219.71 ± 3.90[d]	7.68 ± 0.61[e]
Ascorbic acid ([*]4.5 µg/mL)	1.70 ± 0.01[e]	69.91 ± 2.05[*]

Different superscript letters in the same column show a significant difference ($p < 0.05$). Data are served as mean ± SD (n = 3)

However, 75% methanol extract of *G. spinosum* showed the highest radical scavenging compared to 100% methanol extract of *G. spinosum*, whereas the aqueous extract of *G. spinosum* revealed low scavenging activity. DPPH scavenging activity increased with the increasing of the methanol composition in preparation of extracts. Ascorbic acid, as the positive control, depicted a maximum scavenging effect at a low concentration (4.5 µg/mL). However, there was a significantly high difference ($p < 0.05$) between % inhibition of these extracts.

Reducing sugar and total phenolic contents correlation

The correlation between reducing sugar and total phenolic contents is stated in Figure 2. Results of this study demonstrated that the values of R and R^2 were 0.987 and 0.975, respectively. These scores indicated that reducing sugar content positively correlated with total phenolic content. The statistical analysis proved a significant relationship between these two parameters ($p < 0.05$). The result suggested that sugar content was linked to the total phenolic content. In plants, the glycolysis and pentose phosphate pathways potentially serve as a source of carbon to produce secondary metabolites, such as phenolic compounds.[32] In the shikimic pathway, sugar molecules play an important role to form phenolic compounds. Previously done studies also reported the reducing sugar content and total phenolic compounds correlations.[33-35]

Reducing sugar content and reducing power correlation

Figure 3 shows the correlation between reducing sugar content and reducing power correlation. There was a strong correlation found between the amount of sugar-reducing content and antioxidant activity ($R = 0.991$, $R^2 = 0.982$). The total phenolic content and reducing power found to have a positive and significant ($p < 05$) correlation, according to the data analysis. It implies that plant samples' overall phenolic content may be a factor in the antioxidant activities of those samples. Other similar investigations had also proven that the total phenolic content positively correlates with antioxidant activities.[33,36-37]

Total phenolic content and DPPH correlation

The correlation between total phenolic content and DPPH as summarized in Figure 4. The values of determination coefficient (R^2) and Pearson's coefficient correlation were detected as 0.930 and 0.965, respectively. Further, the data analysis revealed that TPC positively correlated with DPPH. This result suggested that TPC had a potential role in antioxidant activities. The result of this study was found to be supported by some previous similar studies, which had also reported positive relationships between TPC and DPPH.[38-40]

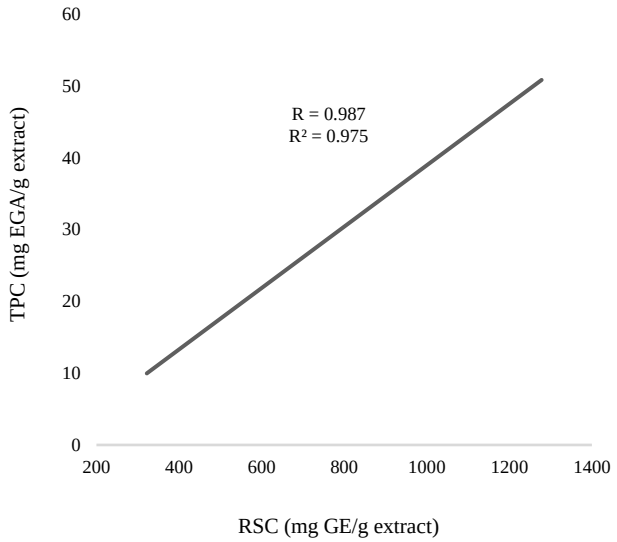

Figure 2: Correlation between RSC and TPC

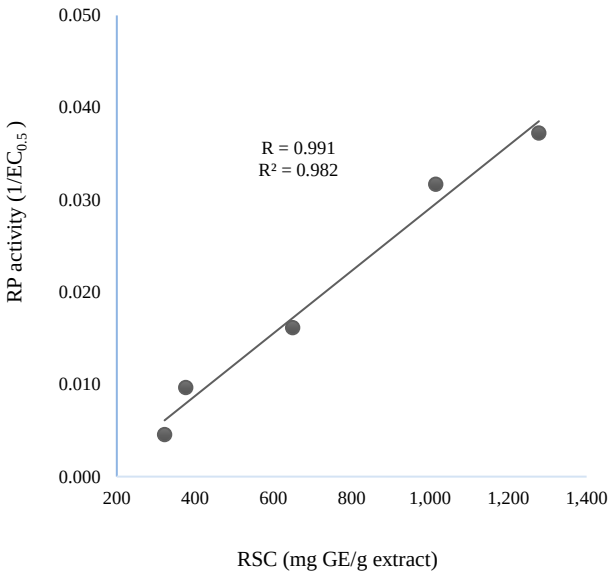

Figure 3: Correlation between RSC and RP activity

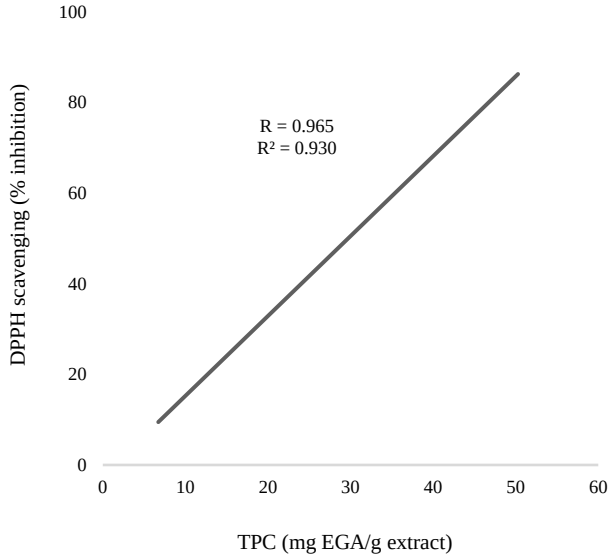

Figure 4: Correlation between TPC and DPPH

Conclusion

The 100% methanol extract of *G. spinosum* was rich in alkaloids, flavonoids, glycosides, polyphenols, proteins, reducing sugar, saponins, steroids and tannins. The aqueous extract of *G. spinosum* contained flavonoids, glycosides, polyphenols, reducing sugars, saponins, and tannins at a moderate level. The highest total phenolic and reducing sugar contents were found in 100% methanol extract of *G. spinosum*. The highest antioxidant activity was found in the 100% methanol extract of *G. spinosum* for reducing power assay, while for DPPH method was found in 75% methanol extract of *G. spinosum*. The study has also proven positive correlations between (i) RSC and TPC, (ii) RSC and RP, and (iii) TPC and DPPH. The findings of this study further support the nutraceutical potential of *G. spinosum*.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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