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THE COMPARISON OF SPECTROPHOTOMETRIC AND TLC-DENSITOMETRIC FOR DPPH RADICAL SCAVENGING ACTIVITY ANALYSIS OF THREE MEDICINAL PLANT EXTRACTS

Christina Setyadewi M., M. Suzery, Agustina L. N. Aminin, and Bambang Cahyono*

Department of Chemistry, Faculty of Sciences and Mathematics, Universitas Diponegoro
Jl. Prof. Soedarto S.H., Semarang, Central Java, Indonesia

* Correspondence: phone/fax : 024-7460033, email: cahyono@live.undip.ac.id

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ABSTRACT

In this research, Thin Layer Chromatography-Densitometry has proven to be a good method for analyzing 1, 1 diphenyl 2-picrylhydrazyl (DPPH) radical scavenging activity, since this approach displayed the similar trends with UV-Vis spectrophotometric method. Three medicinal plants collected from Semarang used to evaluate both methods. The IC_{50} value ranged from 25.26 - 4913.74 ppm shown by UV-Vis spectrophotometric and 24.74 – 4674.61 ppm using TLC-Densitometric. Meniran Dechlorophyllated (*Phyllanthus niruri)* provides the strongest antioxidant activity and the weakest de-chlorophylated parsley *(Petroselinum crispum*). The paired sample t-test points from a non-dechlorophylation extract using the TLC densitometry test significantly gives a lower IC50 value than the UV spectrophotometry method-has. The maximum increase of peak area under the UV light 365 nm up to 56.08 %. This evidence supports the presumption that the scavenging radical DPPH caused not only decreasing the maximum absorbance under UV light 516 nm but also increasing the absorbance under UV light ± 365 nm.

***Keywords**: spectrophotometric; TLC-Densitometric; scavenging DPPH)*

INTRODUCTION

Meniran (*Phyllanthus niruri*), parsley (*Petroselinum crispum*), and kersen (*Muntingia calabura*) were three plants that potentially can be developed as medicine resources related to antimicrobial agent [1,2,3], anti-carcinogenic [4, 5], anti-diabetic [6,7,8]. Parsley is a popular vegetable and spice in Europe. It is widely spread and easy to grow [9]. Apigenin, as the flavonoid of this plant, has been shown to act as a free radical In relevant studies, 50 mg/kg of apigenin was given i.p. for female Sprague Dawley rats for 21 days [10,11]. Anthracene-induced mammary tumors in Sprague Dawley mice, this treatment can prevent the accelerated development of 7,12-dimethylbenz (a) medroxyprogesterone acetate. while 20 mg/kg of apigenin is given i.p. for C57BL / 6 mice showed an anti-tumor effect in malignant mesothelioma caused by transplantation of mice with MM # 40a cells that form ascites [12]. Another report showed decreased carcinogenesis of the large intestine in rats treated with azoxymethane in the diet of male Sprague-Dawley rats where 0.1% apigenin and 0.02% naringenin had been added for ten weeks (Figure.1) [13, 14]

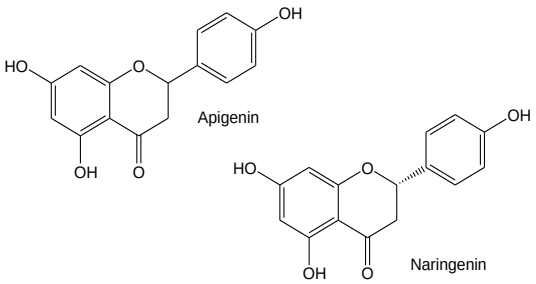

Figure 1. Apigenin and Naringenin

Phyllanthus niruri is distributed in tropical and subtropical regions such as Europe, Central America, Asia (including China, Pakistan, India), West Africa, and South America [15]. P. niruri Linn. scientifically has antioxidants [16], antimalarials [17], anti-hyperuricemic [18], hepatoprotective [19], hypolipemic activity [20]. Chemical compounds caused those pharmacologic effects in meniran, including flavonoid, alkaloid, terpenoid, lignan, polyphenol, tannins, coumarin, and saponin [21].

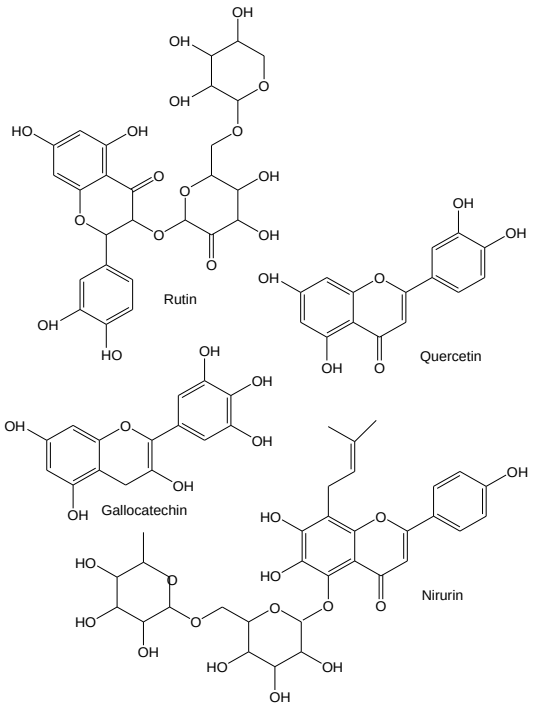

Figure 2. Flavonoid contained in *Phyllanthus niruri* Linn (rutin, quercetin, gallocatechin, nirurin)

The extract potentially contains phenols, tannin, and flavonoids, which provide antioxidant activity in different degrees because of its hydroxyl. Some flavonoids identified in *Phyllanthus niruri* Linn were rutin, quercetin, gallocatechin, nirurin [22]. Flavonoid of *Phyllanthus niruri* Linn. Indicated antioxidant activity, and alkaloid indicated antispasmodic activity (Figure.2) [23]. Previous studies showed the DPPH radical scavenging activity stated as IC_{50} of *Phyllanthus niruri* was 10.53 µg/ml compared to IC_{50} of ascorbic acid 8.90µg/ml [24]. Meanwhile, Rusmana (2017) proved that IC_{50} of *Phyllanthus niruri* was 4.24 µg/ml compared to IC_{50} of quercetin 0.55 µg/ml [25].

Muntingia calabura is native to southern Mexico, Central America, Trinidad, tropical South America, and is also widely cultivated in warm regions in Southeast Asia such as Malaysia, Indonesia, and the Philippines and India. This tree species is one of the public road trees in almost all parts of the world [26, 27]. Identification in *Muntingia calabura* using GC-MS yielded 14 compounds, including geraniol (26.335%), citronellol (16.958%) and eugenol (1.950%). Identification using LC-MS indicated that there were gallic acid (18.607%), catechins (14.077%), quercetin (10.255 %), ellagic acid (9.626%) and kaempferol (8.699%) [28]. Some studies reported bioactivities of *Muntingia calabura* as antioxidant, anti-diabetic, anti-microbial, anticancer, anti-inflammatory. The methanol extract of its fruit showed the highest antioxidant activity compared to its extract using petroleum ether, chloroform, ethyl acetate, and butanol [29]. The IC_{50} value of ethanolic extract of *Muntingia calabura* leaves (DPPH method with BHA control) was 79.96 ± 0.91 µg / mL [30]

Three medicinal plants have potential as medicinal resources, and comparative studies of antioxidant activity of three medicinal plants have never existed. Several traditional medicinal plants have been reported to scavenge DPPH radical [2, 31, 32]. There is a positive correlation between anti-proliferative and DPPH radical scavenging activity of trihydroxyflavone determined for A549 and U87 cancer cells. Trihydroxy-flavone demonstrated anticancer activity. All studies used plant extract. However, there were some laboratory experiences supported by literature showing pigment contained in plants, especially in leaves, often affected the quality of secondary metabolite analysis [33]. The chlorophyll that has absorbance and fluorescence emission spectra in 350 – 450 nm [34, 35] may affect antioxidant activity assay. Several analytical techniques are currently used for antioxidant activity assay, including spectrophotometric determination and liquid chromatography analysis. Some studies concerning the analysis of DPPH radical scavenging of secondary metabolite compounds using the UV-Vis spectrophoto-metric method showed interferences by the compounds contained in the extract samples that absorb in the visible area light [36,37,38, 39]. Therefore, to provide a good, accurate, and simple method for determining antioxidant activity, research on the comparison UV-Vis Spectrophotometric and TLC Densitometric method is necessary.

METHODS

In this report, the cleaning activity of DPPH radicals via UV-Vis spectrophotometer for UV analysis and TLC for chromatography.

1. Materials

Meniran (Phyllanthus niruri), Parsley (Petroselinum crispum), and *Kersen (Muntingia calabura* L) were collected from Semarang. 1, 1 diphenyl 2-picrylhydrazyl (DPPH), ethanol, methanol, chloroform, aluminium plates precoated with silica gel 60 F_{254}.

2. Preparation of Extracts

Phyllanthus niruri, Petroselinum crispum, and *Muntingia calabura* L were washed with water to remove dust particles, then dipped in hot water for 10 seconds. The washed leaves were shade dried at room temperature. The dried leaves were extracted by maceration method with ethanol (96%) as a solvent. Dechlorophyllation of the plant extracts was conducted in accordance with an adaptation of the methodology used by Sando [40]. Half of the initial volume of extract solution from maceration was evaporated, then hot water was added. After the mixture reached room temperature, ethyl ether was added. The fraction containing chlorophyll was at the top. Therefore, the bottom part could be separated using a separatory funnel. The drying process was continued by a rotary evaporator and freeze dryer. Dechloro-phyllated extract samples would be mentioned as meniran a parsley a, and kersen a. And the non-dechlorophylated extract samples would be mentioned as meniran b, parsley b, and kersen b.

3. DPPH Scavenging Activity Assay

UV-Vis Spectrophotometric Method. The Measurement of antioxidant activity of plant extracts using 2, 2 diphenyl 1-picrylhydrazyl (DPPH), radical scavenging activity was carried out by adapting the

methodology described by Molineux [41]. Use Three and a half mL DPPH 25 ppm in methanol and mix with 0.5 ml of plant extracts starting from 4, 8, 12, 16, 20 µg / mL where further incubation [there is room temperature for 20 minutes. The optical density of the reaction mixture was taken at 517 nm. Capability is calculated as:

DPPH scavenging activity (%) =

$$[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \quad (1)$$

Where $Abs_{control}$ is the absorbance of DPPH + methanol; Abs_{sample} is the absorbance of DPPH + sample (Extract/Quercetin)

Thin Layer Chromatography Densitometric Method. A Camag TLC Scanner 3 and VisionCATS 2.5 software were used to scan and measure the absorbance/reflectance of each sample. Plate development was carried out in a chromatographic chamber (20 cm x 20 cm). Chromatographic plates were cut 8 cm x 7 cm from 20 cm x 20 cm aluminium plates precoated with silica gel 60 F_{254} (E. Merck, Germany). Spotting of samples used micropipettes (1 μL, Camag).

DPPH radical scavenging activity analysis using the TLC Densitometric method was conducted withby, an adaptation of the methodology described by Abourashed [42]. Before each test, prepare a DPPH solution in methanol (0.5 mg / 5 mL, 250 µM), A standard methanolic solution of the reference antioxidant quercetin, was prepared. It was serially diluted with methanol to obtain a set of 5, 10, 15, 20 and 25 ppm. Meanwhile, each sample was also prepared and serially diluted with methanol to obtain sets of kersen a (40, 60, 80, 100 and 120 ppm), kersen b (20, 40, 60, 80 and 100 ppm), meniran a (20, 40, 60, 80 and 100 ppm), meniran b (20, 40, 60, 80 and 100), parsley a (2000, 4000, 6000, 8000 and 10.000 ppm) and parsley b (1000, 2000, 3000, 4000 and 5000 ppm). A 1 µL of each sample and quercetin standard were mixed with 1 µL DPPH solution. After 20 minutes, the mixture was applied in triplicate to a TLC plate. After developing the spotted plates in a chamber with a mobile phase solution, the plates were scanned at 516 nm, and their peak areas recorded.

RESULTS AND DISCUSSION

The UV-Vis spectroscopy technique is the simplest technique for identifying samples based on how much the substance absorbs light by measuring the intensity of the light when a beam of light passes through the sample solution.The spectrum of all extracts, standard compound quercetin, and DPPH used in this research displayed in Figure 3.

The *UV-Vis* flavonoid spectra consist of maximum absorbance in the range of 240 to 290 nm (*band* II: benzoyl band) and the range 300-550 nm (*band* I: cinnamoyl band) [43,44]. All extracts and quercetin displayed maximum absorbance in the range of 240 to 290 nm, which indicated they all had the benzoyl band. DPPH, quercetin, the extract of parsley kersen also showed the cinamoil band as they displayed maximum absorbance in the range of 300 to 550 nm. DPPH pointed the highest spectra with maximum absorbance at 516 nm. But meniran extract did not show maximum absorbance in the range of 300 to 550 nm.

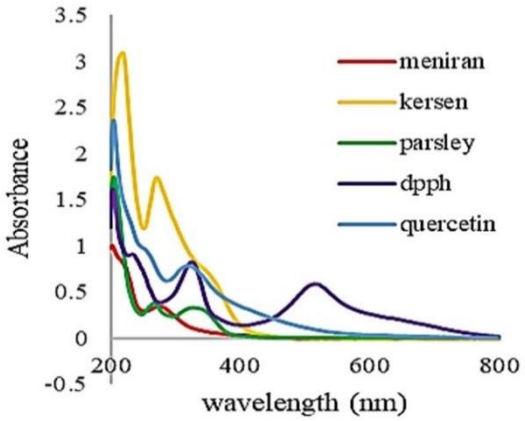

Figure 3. The spectrum of all extracts, standard compound quercetin, and DPPH

DPPH (α,α-difenil-β-- picrylhydrazyl) is a stable free radical. The delocalized electrons with seven conjugated double bonds gave a deep purple color with absorption in a methanol solution of about 516 nm. Radical DPPH reacted to antioxidant compounds to produce new bonds, thus changing the color of the solution. The reduction in purple intensity was caused by a decrease of the chromophore or a conjugated double bond in DPPH. This reactivity has been used to test the ability of meniran, kersen, and parsley extracts to scavenge DPPH free radicals. DPPH radical reduction was monitored by spectrophotometry as a decrease in absorbance at 516 nm (Figure 4.)

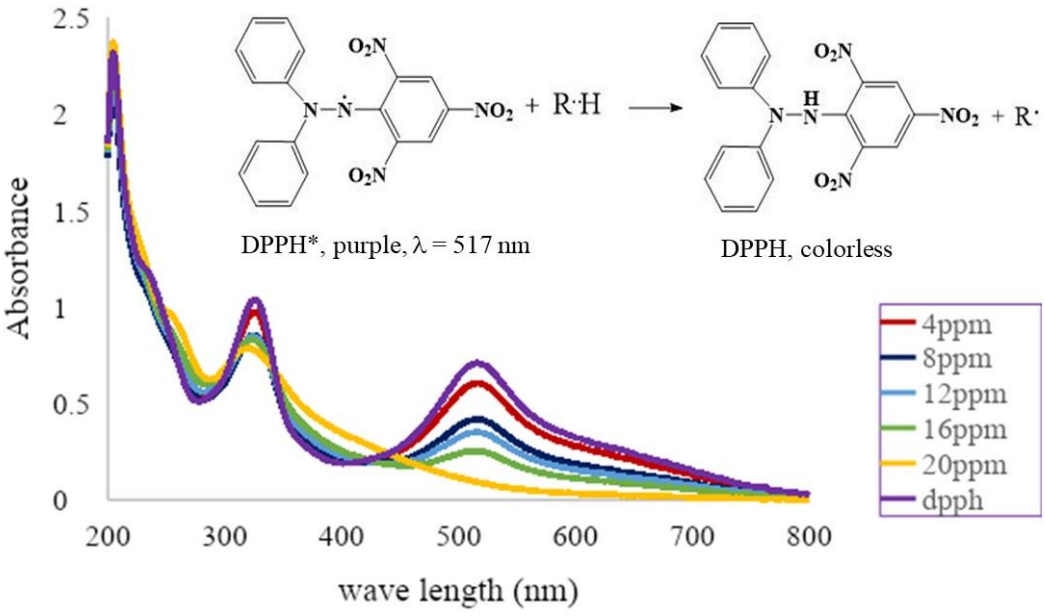

Figure 4. DPPH radical reduction by quercetin as an antioxidant compound

A comparison of IC_{50} value using UV spectrophotometric and TLC densitometric method is presented in Table 1. The radical scavenging activity displayed in Table 1 shows that standard compound quercetin has IC_{50} 11.62 + 0.175 ppm, which was relatively close to the previous report that stated the IC_{50} of quercetin was 9.0 ± 0.1 [45]. The DPPH radical scavenging activity of various extracts of dechlorophyllated and non-dechlorophyllated meniran and kersen had good DPPH radical scavenging. Among the four samples, dechlorophyllated meniran extract had the strongest activity of DPPH

radical scavenging (IC_{50} = 25.26 ± 0.036). Compared to the IC_{50} of quercetin, dechlorophyllated meniran extract was relatively strong whereas the IC_{50} of parsley was far above 100 ppm, which was categorized as a very weak DPPH radical scavenging activity. As mentioned above that meniran did not show absorption in 300–550 nm (cinnamoyl band), apparently this did not affect the ability to scavenge DPPH radical.

Table 1. Comparison of IC_{50} value obtained using UV spectrophotometric and TLC densitometric method

Extract Sample	IC_{50} value ± SD (ppm)			Maximum increasing peeak area under UV light 365nm
	Reference (spectrophotometric metode)	Spectrophotometric method	TLC densitometric method	
Meniran A		25.26 ± 0.036	24.74 ± 1.61	51.92 ± 2.97 %
Meniran B	14.5 [45]	63.93 ± 0.0028	37.51 ± 3.03	28.15 ± 2.61 %
Kersen A		72.73 ± 0.049	32.83 ± 3.96	26.75 ± 4.57 %
Kersen B	79.96 ± 0.91 µg/ml [30]	117.6 ± 0.0361	46.49 ± 1.86	41.52 ± 1.48 %
Parsley A		4913.74 ± 1.588	4674.612 ± 960.385	56.08 ± 3.88 %
Parsley B	3310.0 ± 80.5 µg /mL [46]	3247.36 ± 1.642	1481.24 ± 121.06	26.19 ± 3.37 %
Quercetin	9.0 ±0.1 [42]	11.62 + 0.175	6.51 ± 1.07	41.87 ± 2.65

Note: meniran A, kersen A and peterseli A = dechlorophylated extract samples
meniran B, kersen B and peterseli B = non dechlorophylated extract samples

Densitometric measurements transformed the substance distribution on TLC plates into digital computer data [47]. The Measurement is based on the optical density in a light-sensitive plate. The mobile phase selection became an important step in the TLC Densitometric method. It was based on adsorbent material used and the physical-chemical properties of the analyte. The more non-polar the compound, the faster it eluted, or less time it remained on the stationary phase [48]. The mobile phase, which gave the best separation of all compounds in kersen and meniran extract, was chloroform. Meanwhile, the mobile phase for parsley was a solution mixture of chloroform: methanol = 4.5: 0.5. Chloroform is a semi-polar eluent, while methanol is a polar eluent. A good separation (Figure 5) was shown by kersen extract, which was eluted by chloroform with R_f values of the most distant compound is 0.58. It means kersen contains compounds which have characteristics between semi-polar and polar. Whereas meniran and parsley only showed a not very clear separation despite the eluent being a mixture of semi-polar and polar eluent (chloroform: methanol)

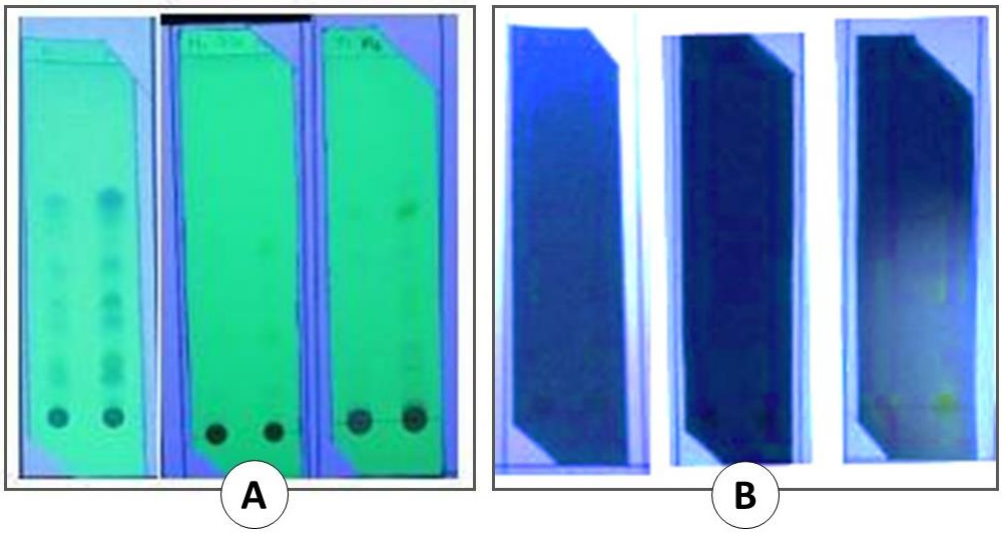

Figure 5. TLC after development of kersen (a & b) using chloroform solvent, meniran (a & b) and parsley (a & b) using mixture of chloroform: methanol (4,5 : 0,5) solvent, scanning under UV light 254 nm (A) and 365 nm (B)

The free radical scavenging activity was reflected in a progressive drop in the optical density spots at 516 nm after elusion. It was close to the absorbance wavelength range (510–520 nm) reported in the literature for DPPH solutions using UV-Vis Spectrophotometer [49]. Spots before and after elusion showed different forms of peaks. The peak was not well integrated before elution, but the integrated peak formed after elution then can be calculated (Figure 6). The values found a parallel with the increasing concentration of each sample. The higher the concentration, the peak area got smaller. Premixing DPPH and the sample extracts as antioxidant compounds and allowing the reaction to proceed in solution followed by application and Measurement on the TLC plate proved to be reproducible. Therefore, the approach of premixing was adopted. The DPPH radical scavenging activity obtained under the TLC assay showed a similar trend as obtained by UV spectrophotometric. It was also in line with data reported elsewhere. Akar et al. [50] performed a related study, using the TLC plate to carried out antioxidant assay with DPPH radical applied on synthetic and natural antioxidants and medicinal herbs. The spots on the TLC plate, after the incubation period, will emerge and then were evaluated with Image J software to determine the CSC_{50} value. This study showed that a 50% color reduction happened in the sample concentration, which was very similar to the SC_{50} value obtained by the spectrophotometric method. Furthermore, color measurement using a smooth surface (TLC or paper). a scanner and the free downloadable colour measurement software Image J. A, Abourashed introduces a quantitative method based on densitometric evaluation of dry spots [50] as a substitute to the current absorbance based wet methods for the quantitative estimation of DPPH scavenging activity. It said that the coefficients of variation for all IC50 values were around 5%, which denoted method reproducibility and the suitability of the used technique for the intended resolve. Although

the deviation standard is relatively bigger than the data obtained using UV Spectrophotometric, it is still relatively close to the reported study 9.0 ± 0.1.

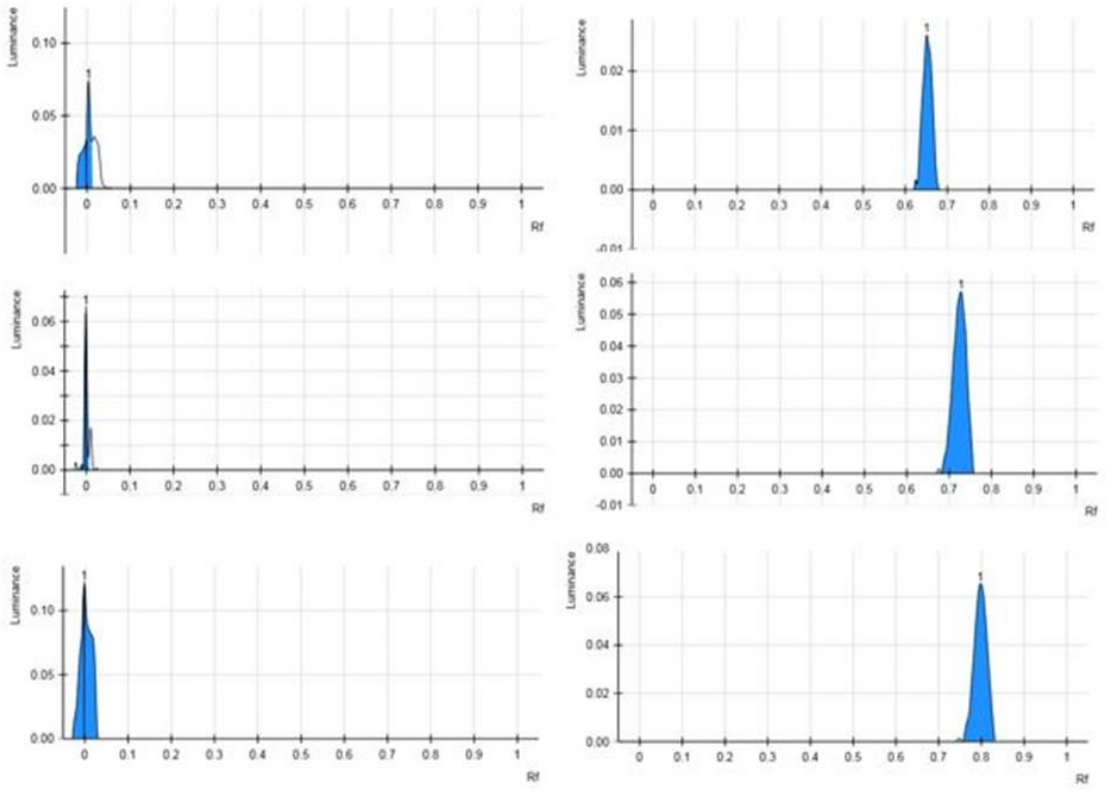

Figure 6. Chromatograms of the extract samples reacted with DPPH and analyzed at 516 nm wavelength, before (A) and after (B) development using chloroform as the mobile phase.

The observation of peak area under UV light 365 nm showed the tendency to increase up to 56.08 %. It could be said that the scavenging radical DPPH process caused not only decreasing maximum absorbance under UV light 516 nm but also increasing absorbance under UV light ± 365 nm. The important and interesting point is that the IC_{50} values obtained using TLC densitometric assay were lower than IC_{50} values obtained under UV spectrophotometric. It might be because TLC densitometric only measured the compound, which scavenged DPPH radical without any interference of other compounds. The high specificity of TLC techniques has been reported by several studies. The TLC method with densitometric exposure was recognized for the quantification of p-chlorophenol in wastewater [52]. The nearly identical results achieved using TLC and HPLC, led to the deduction that both approaches could be applied for such examinations. Dolowy et al. [53] also report the development of TLC-densitometry for the simultaneous purpose of hydrocortisone acetate and lidocaine hydrochloride in combined pharmaceutical preparation. The results of hydrocortisone acetate and lidocaine hydrochloride obtained from inspected marketable products matched

with the value given by the company are reliable with those which are recommended by the Polish and United States Pharmacopoeias.

CONCLUSION

In conclusion, this study has indicated that quantification of DPPH radical scavenging activity using UV spectrophotometric and TLC densitometric methods has different results. However, the DPPH radical scavenging activity obtained under TLC densitometric assay showed a similar trend as data obtained under the UV spectrophotometric method. The scavenging activity obtained by UV spectrophotometric method is relatively higher than the TLC densitometric method.

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