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FTIR-based Fingerprinting Combined with Chemometrics for Discrimination of *Sonchus arvensis* leaf Extracts and Correlation with Their Antioxidant Activity

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ABSTRACT

Sonchus arvensis, or known locally in Indonesia as *tempuyung*, is acknowledged to have many biological activities, including antioxidant activity. This study aimed to cluster the leaves extracts based on the extracting solvent and to determine functional groups that significantly contribute to the antioxidant activity. From the water, 10%, 30%, 50%, 70% ethanol, and absolute ethanol extracts, we analyzed the total phenolics content (Folin-Ciocalteu method), antioxidant activity (DPPH method), and the FTIR spectra. The 70% ethanol extract exhibited the highest total phenolics and antioxidant activity. The extracts were grouped based on the extracting solvent using the principal component analysis (PCA) with 95% total variance from its principal component 1 and 2. The partial least square (PLS) regression was employed for finding a functional group that are responsible for the antioxidant activity in the sample extract. PLS regression predicted that the –OH and the C-O groups attributed to the phenolic compounds give a significant contribution to the antioxidant activity of the *S. arvensis* leaf extracts.

Keywords: antioxidant, chemometrics, FTIR spectra, *Sonchus arvensis*, total phenolic content

INTRODUCTION

Sonchus arvensis, or *tempuyung* in Indonesian, is a medicinal plant that belongs to the Asteraceae family and mainly grows wild. *S. arvensis* has been widely used as traditional medicine and vegetable in Indonesia and China. Traditionally, it is used to treat bladder stones, abscesses, cough, asthma, fever, and inflammation, and for detoxification and improving blood circulation (Sukmayadi *et al*., 2014; Li *et al*., 2018). *S. arvensis* is known to have some biological activities such as antioxidant, antibacterial, anti-fatigue, hepatoprotective, kidney-protective, and inhibition of the xanthine oxidase enzyme (Xia *et al*., 2011; Khan 2012; Alkreathy *et al*., 2014; Yuan *et al*., 2019; Trivadila *et al*., 2020). Various active components are present in *S. arvensis* such as phenolic acids, flavonoids, sesquiterpene lactones, steroids, glycerates, and quinic acid esters (Li *et al*., 2018). The phenolic acids and flavonoids include gallic acid, orientin, routine, luteolin, kaempferol, myricetin, catechins, and quercetin (Khan 2012; Seal 2016).

Phenolic acids and flavonoids are known to have significant antioxidant activity. The biological activity level depends on the composition and concentration of the bioactive compounds present in medicinal plants. Location of growth and climatic conditions (such as soil texture, moisture, and light intensity), harvesting, processing, storage, part of the plant, and extraction methods will affect the composition and concentration of extracted metabolites (Verawati *et al*., 2016; Tanamal *et al*., 2017). Also, the type and concentration of the extracting solvent can affect the level of extracted metabolites. Water, ethanol, and their mixtures are

primarily used to extract polar compounds such as phenolics, and these solvents are also good in extracting metabolites (Boeing *et al*., 2014).

There has been a growing interest to use metabolomics for evaluating the effect of various extracting solvents on the extracted metabolites. Based on the quantity of data and the number of detected metabolites, the metabolomics can be classified into three categories: targeted metabolite analysis, metabolite profiling, and metabolite fingerprinting. The metabolite fingerprint analysis is often used to evaluate the metabolites' changes in various circumstances (Sajak *et al*., 2016). The metabolite fingerprint analysis indicates all metabolites' signals in a sample and can be correlated with a biological activity to predict the functional groups belonging to a compound that contributes to the biological activity. Metabolite fingerprint analysis can use data such as FTIR spectra or chromatograms of the total composition without identifying the individual constituent (Krastanov, 2010).

FTIR spectroscopy offers many advantages and meets efficient analysis criteria, such as being easy-to-use, non-destructive, fast, and inexpensive (Bunaciu *et al*., 2011). FTIR spectra profile is a very complex and direct visual inspection to see changes in the number of extracted metabolites is not easy; thus, it requires the help of a chemometric analysis to determine these changes (Gad *et al*., 2012). The combination of FTIR spectra and chemometric analysis such as principal component analysis (PCA) for grouping plant extracts with various extracting solvents has been carried out for legumes (Diblan *et al*., 2018), *Smallanthus sonchifolius* (Aziz *et al*., 2020), and *Guazuma ulmifolia* (Rafi *et al*., 2020).

A combination of FTIR spectra and partial least square (PLS) regression could predict functional groups that have a significant role in certain biological activities, such as antioxidants. This combination has been used for the characterization and identification of functional groups of compounds that correlate with α-glucosidase inhibition of *Salacca zalacca* (Saleh *et al*., 2018) and *Tetracera scandens* (Nokhala *et al*., 2020), also antioxidant activity of *Momordica charantia* fruit (Khatib *et al*., 2017). Juliani *et al*. (2016) also reported a correlation between the antioxidant activity and α-glucosidase inhibition from the extract and fraction of *Orthosiphon aristatus*. However, there has been no report on clustering *S. arvensis* leaf extracts using FTIR spectra and chemometrics analysis and correlation with their antioxidant activity. Therefore, this research aimed to classify *S. arvensis* extract using different extracting solvents and to predict functional groups from extracted metabolites that have significant antioxidant activity using FTIR spectra combined with chemometric analysis. In this study, we macerated the sample with ethanol, water, and ethanol-water mixtures to analyze the effect of different extracting solvents on the extracted metabolites. Principal component analysis was applied to see the clustering of the extracts based on the different extracting solvent, while partial least square was applied to predict functional groups of the antioxidant compounds present in the extracts.

METHODS AND MATERIALS

Tropical Biopharmaca Research Center (TropBRC) IPB University, Bogor, Indonesia, provided the leaf samples. After identifying the samples in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, we stored the voucher specimens in TropBRC. Ethanol was purchased from Merck (Darmstadt, Germany). Gallic acid, Folin-Ciocalteu reagent, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, and potassium bromide spectroscopy grade were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation and extraction

The samples were cleaned from the soil and other impurities by a thorough wash with water, dried in a 40°C in the oven, and pulverized. About 10g of sample powder was added with 150mL of water and macerated for 48h with occasional stirring. We performed the extraction in triplicates. Other extracting solvents used were ethanol in different concentrations: 10%, 30%, 50%, 70%, and absolute, using a similar procedure to that for extraction using water. The filtrate was collected, concentrated using a rotary evaporator, and freeze-dried. The dried extracts were then ready for further analysis.

Determination of total phenolic content

Determination of the total phenolic content followed the Folin Ciocalteu procedure. We used the procedure described by Kruawan and Kangsadalampai (2006). About 10μL of the extract was added with 160μL distilled water, 10μL of 10% Folin Ciocalteu reagent (dissolved in distilled water), and 20μL of 7.5% $NaHCO_3$. After incubating the mixtures for 30min in a dark room, the

absorbance was measured at 765nm in a microplate reader (Epoch-Biotek, Winooski, USA). The calibration curve was established with a concentration series of gallic acid in ethanol at 10, 30, 50, 70, 100mg/L. The total phenolic content was expressed as gallic acid equivalent (GAE) in dried powder sample weight (mg GAE/g dried powder).

Determination of antioxidant activity

DPPH method was used to determine antioxidant activity using a procedure used by Rafi *et al*. (2018). Approximately 200µL of the extract solution was taken and put into 96-well plates and added with 100µL DPPH 0.3mM, then incubated for 30min in a dark room. The absorbance of the mixture was later measured using a microplate reader (Epoch-Biotek, Winooski, USA) at 517nm. We used a concentration range between 5 and 150µg/mL for the extract solutions and ascorbic acid as a positive control. The antioxidant activity was reported as IC_{50} based on the calculation using the correlation curve between the concentration and the percentage of inhibition on DPPH radicals.

Acquisition of FTIR Spectra

Approximately 2mg of sample extract was mixed with 200mg KBr and pressed into the disc using a manual press for 10min. The disc was placed in the Tensor 37 FTIR spectrophotometer (Bruker Optik GmBH, Ettlingen, Germany). Deuterated triglycine sulfate (DTGS) was used as the detector. FTIR measurement was made in the region of 4000–400cm^{-1} with a resolution of 4 cm^{-1} and 32 s/min operated by OPUS 4.2 software (Bruker Optik GmBH, Ettlingen, Germany). FTIR spectra were stored as a data point table.

Data analysis

The experimental data were collected in triplicate measurements and reported as mean ± standard deviation. Statistical comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Duncan test, with a significant difference was defined at the 95% confidence level ($p<0.05$).

Before being used to cluster the sample extracts and correlate with the antioxidant activity, FTIR spectra were preprocessed using standard normal variate (SNV). The absorbance data used for grouping the extracts were within 3200-2800cm^{-1} and 1800-400cm^{-1} regions. Clustering the extracts according to the extracting solvents was performed using PCA.

Identification of functional groups that gave a significant contribution to the antioxidant activity was performed using PLS regression analysis by taking a correlation between the FTIR spectra and the IC_{50} values of the antioxidant activity. We employed Unscrambler X version 10.1 (CAMO, Oslo, Norway) for running the PCA and PLS regression analysis.

RESULTS AND DISCUSSION

Yield of extracts

The yields from six different extracting solvents: water, 10%, 30%, 50%, 70% of ethanol, and absolute ethanol (Table I). Maceration in 10% ethanol gave the highest yield (21%), followed by water, 30% ethanol, 50% ethanol, 70% ethanol, and ethanol. The obtained result indicated that phytochemicals present in *S. arvensis* are polar to semi-polar. We found that from the analysis of variance, different extracting solvents affected the extract yields. The yields of water extract and 10-50% ethanol extracts were not significantly different ($p<0.05$), while those from 70% ethanol and absolute ethanol differed significantly from the more dilute ethanol extracts. This result suggests that the type and the concentration of the extracted compounds depend on the extracting solvent.

Total phenolics content

Phenolic compounds in a plant may include phenolic acids, flavonoids, anthocyanins, lignin, and tannins. Phenolic compounds are the predominant secondary metabolites in terrestrial plants. The phenolic compound is generally known to have antioxidant properties through reduction, free radical scavengers, metal chelators, and electron donor mechanisms (Camargo *et al*., 2016). However, not all phenolic compounds have potential antioxidative properties (Haq *et al*., 2011). Khan (2012) reported that *S. arvensis* leaves have antioxidant compounds that belong to the phenolic class.

Total phenolic content was determined using the Folin-Ciocalteu method. Folin-Ciocalteu reagent reacts specifically with the phenolic compounds. The reagent, which was originally yellow, turns blue upon reaction as the aromatic rings of the phenolic compounds reduce phosphomolybdate-phosphotungstate in the Folin-Ciocalteu reagent to blue molybdenum (Buyuktuncel *et al*., 2014).

Table I. Extraction yields, total phenolic contents, and antioxidant activities of *S. arvensis* extracts

Extracting Solvent	Extraction yield (%)	Total Phenolic Content (mg GAE/g dried powder)	Antioxidant activity (IC_{50}, µg/mL)
Distilled water	20.35^{d}±1.39	59.751^{a}±3.86	133.746^{f}±0.60
10% Ethanol	20.51^{d}±0.33	134.540^{c}±0.64	119.060^{e}±1.99
30% Ethanol	17.35^{c}±1.53	87.750^{b}±1.25	98.804^{d}±1.21
50% Ethanol	15.92^{c}±1.23	252.952^{d}±0.20	89.371^{c}±0.74
70% Ethanol	9.21^{b}±0.42	269.453^{e}±14.19	22.017^{a}±2.03
Absolute Ethanol	6.73^{a}±1.76^{a}	216.819^{c}±3.78	29.576^{b}±2.03

The reported values are mean ± SD of the triplicate assay for each sample. The mean ± SD within each extract in the same column followed with different subscript letters represent significant differences at $p < 0.05$

Table II. The identified functional groups in *S. arvensis* leaves extracts

Wavenumber (cm^{-1})	Functional Groups
3500–3300	–OH
2930–2820	–CH sp^2
1740–1720	–C=O aldehyde
1600–1500	–C=C aromatic
1467	–CH sp^3
1380–1270	–OH phenolic
1027–1070	–CO
735–727	–CH aromatic

Determination of the total phenol levels is based on the ability of the phenolic compounds to prevent oxidation reactions; hence it is suspected that there is a close relationship between the total phenolic contents and their antioxidant activity (Djapiala *et al*., 2013).

The extracts' total phenolic contents ranged from 60 to 270 mg GAE/g dry extract (Table I), with the highest was in the 70% ethanol extract. The total phenolic contents from the highest to the lowest were 70% ethanol > 50% ethanol > ethanol > 10% ethanol > 30% ethanol > water extracts. The extracts' total phenolic contents showed a significant difference between solvents at a 5% confidence level. Based on these results, 70% ethanol is the most efficient solvent for extracting phenolic compounds from *S. arvensis* leaves.

Antioxidant activity of *S. arvensis* extracts

DPPH method offers some advantages for determining antioxidant activities of the extracts, such as rapid yet straightforward analysis and provides information on the sample reactivity towards stable DPPH radicals. Antioxidants' ability to scavenge the DPPH radicals is known from the change of the purple DPPH color to yellow due to the electron transfer reaction. In this study, we reported the level of antioxidant activity as IC_{50}, with the low IC_{50} indicates high antioxidant activity.

The extracting solvent gave a significant difference in the antioxidant activity level (Table I). We found the highest antioxidant activity was from 70% ethanol extract with an IC_{50} of 22µg/mL, followed by 50%, 30%, 10% ethanol, and water extracts. In other words, by using 70% ethanol, we could get greater amounts of extracted antioxidant compounds than other extracting solvents. Furthermore, all extract antioxidant activities were significantly different among solvents at a 5% confidence level, meaning that all extracts might contain a different concentration of antioxidant compounds. Hence, using extracting solvents with different polarities could give different levels of antioxidant activity.

Molyneux (2004) classifies antioxidant activity based on the IC_{50} as follows: very strong if the IC_{50} <50 µg/mL, strong if in the range 50µg/mL < IC_{50} <100 µg/mL), moderate if 100µg/mL < IC_{50} > 150 µg/mL, weak if 150µg/mL <IC_{50} >200µg/mL, and very weak if the IC_{50} > 200 µg/mL. According to this classification, the antioxidant activities of the extracts were moderate to very strong.

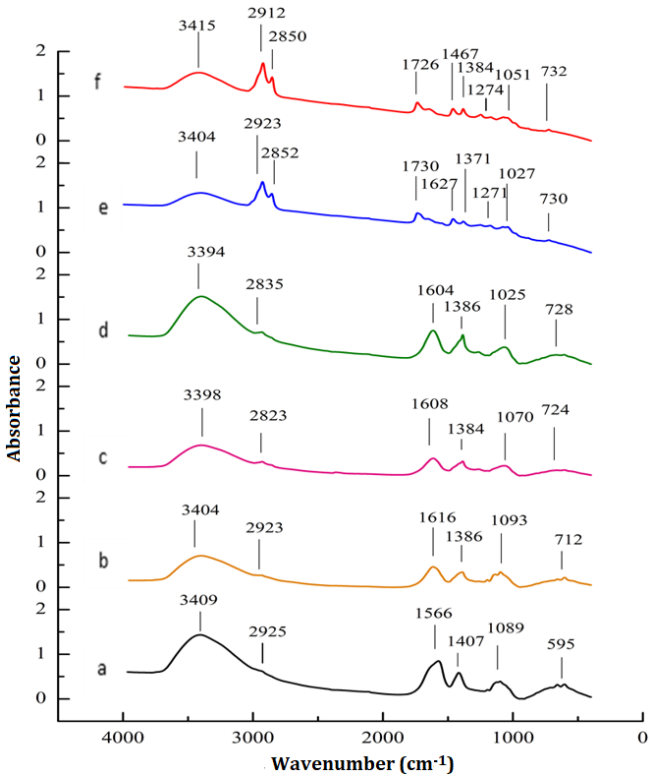

Figure 1. FTIR spectra of *S. arvensis* leaf extracts with six different extracting solvents, (a) water, (b) 10% ethanol, (c) 30% ethanol, (d) 50% ethanol, (e) 70% ethanol, and (f) absolute ethanol.

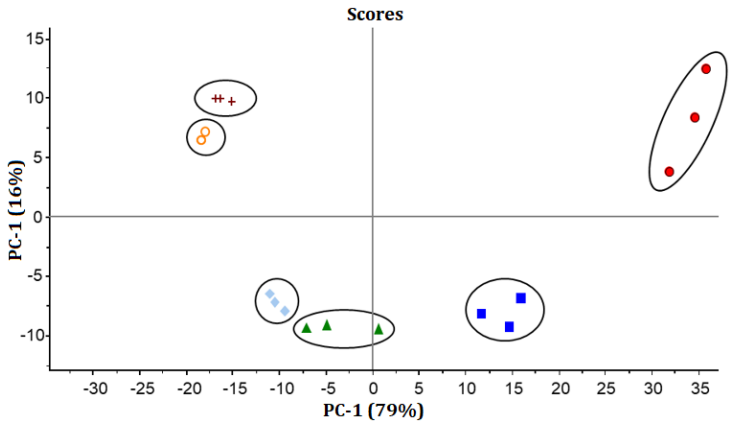

Figure 2. PCA score plot of *S. arvensis* leaf extracts: water (■), 10% ethanol (●), 30% ethanol (▲), 50% ethanol (♦), 70% ethanol (+), and absolute ethanol (o)

The water and 10% ethanol extracts were classified as moderate. The 30% and 50% extracts and the other two extracts (70% ethanol and ethanol) were classified as strong and very strong, respectively.

There is a clear correlation between the total phenol and the antioxidant activity; a high concentration of phenolic compounds correlates positively with the antioxidant activity (Meenakshi *et al*. 2009). The experimental data followed the statement above, showing that the 70% ethanol extract had the highest phenolic content and the lowest IC_{50} value.

FTIR spectra interpretation

The FTIR spectra provide a distinct profile that describes the signals resulting from the vibration and rotation of extracted metabolites from a sample, also known as fingerprint analysis. Different extracts will display different spectral patterns (Easmin *et al*., 2016). Figure 1 shows the FTIR spectra of all extracts. All of the extracts offer almost similar patterns except that for the absolute and 70% ethanol extracts. The latter two extracts revealed a slightly shifted absorption from the wavenumber of 1726cm^{-1} to 1730cm^{-1}. The noticeable difference for the entire FTIR spectra lied in the absorbance intensity only.

A similar pattern of all FTIR spectra indicates that the extracted metabolite compositions were not significantly different, except for concentration level. The difference in concentration levels will affect its biological activity, such as antioxidant activity. Table II demonstrates the functional groups identified in all extracts. The hydroxy-functional group was identified in all extracts from absorption at a wavenumber around 3400cm^{-1}. The absorption of the phenolic functional group was at 1380–1270cm^{-1} and C–O absorption was within the wavenumber of 1027–1070 cm^{-1}.

Clustering of extract constituents

PCA analyzed the extracts from six extracting solvents to ensure further differentiation in the composition and extracted metabolites' levels. The PCA works by reducing a variable into a new set of variables, called the principal component (PC). This PC is a linear combination of the spectrum data variables and contains the most relevant information (Rachmawati *et al*., 2017). PC is depicted in the form of a score plot, a projection of several objects onto two PCs, and is an excellent way to find similarities or differences between samples (Masoum *et al*., 2014). The score plot shows that the closer a sample is to another, the greater the similarity between these samples' metabolite profiles (Purwakusumah *et al*., 2014). PCs often used in the PCA analysis are the two initial PCs, namely PC1 and PC2. The PC1 is the main component representing the most considerable variance in the data set, while the PC2 is the main component with residual variations based on the PC1 point of view (Masoum *et al*., 2014).

Preprocessing of FTIR spectra is necessary before the PCA analysis. Preprocessing can overcome noise and other interferences on the spectrum data to improve the grouping result accuracy without reducing the analytical information. The standard normal variate (SNV) was used as a preprocessing step in this work. SNV is the most used for spectroscopic data and works by eliminating the scatter effect by centering and scaling the individual spectrum (Kusumaningrum, 2017).

The absorbance data used for clustering the extracts were in the range of 3200–2800cm^{-1} and 1800–400cm^{-1} wavenumbers. The selection of these regions provides specific information on the absorbances, and the 1800–400cm^{-1} regions were a unique fingerprint area for each sample. Figure 2 showed the PCA score plot using two principal components accounting for 95% of the total variance with PC1 and PC2, explaining 79% and 16% of the total variance, respectively. Based on the score plot, all extracts were clustered into their respective groups. This plot clarifies the similarities and differences in the FTIR spectrum of the extract; the closer one group is to another, the greater the similarity in composition and level of extracted metabolites. It shows that the 30% and 50% ethanol extracts, also 70% and absolute ethanol extract were close to one another, suggesting that the levels of extracted metabolites were not varied significantly.

Correlation of FTIR spectra and antioxidant activity

The metabolite functional groups in the extracts that significantly contributed to the antioxidant activity level were determined using a partial least square (PLS) regression. The PLS model was carried out using two data sets: the FTIR spectrum absorbance variable as the independent variable (X) and the IC_{50} value of antioxidant activity as the response variable (Y). The data on PLS were given the same preprocessing treatment as in the PCA. From the PLS regression analysis, a regression coefficient plot was obtained, which provides information on the metabolite functional groups that significantly contribute to the antioxidant activity.

The amount of antioxidant metabolites in the extracts affected the resulting antioxidant activity. The regression coefficient plot (Figure 3) illustrates the functional groups with the most contribution from an antioxidant compound. This plot provides information about the importance of variable X to variable Y. The functional group that contributes significantly will have a negative regression coefficient value (Guo *et al*. 2017).

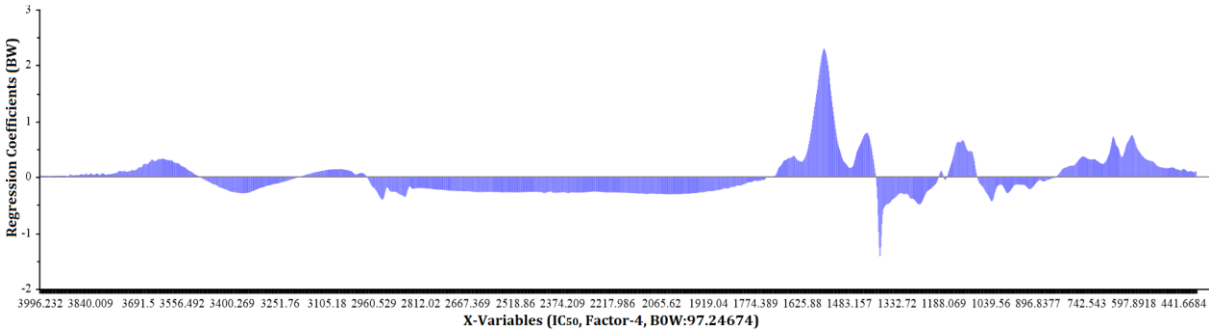

Figure 3. Regression coefficient plot of *S. arvensis* leaves extracts

The functional groups that gave negative values were –OH at wavenumbers of 3500–3300 cm^{-1} and 1205–1124 cm^{-1}, which was the stretching vibration of the C–O bonds from the resulting regression plots. Based on the identified functional groups, namely –OH and C–O, which are typical for phenolic compounds, we suggest that phenolic compounds play a significant role in the level of antioxidant activity.

CONCLUSION

The type of extracting solvent affected the FTIR spectra profile and the antioxidant activity. The spectra were similar amongst the solvents. The 70% ethanol extract gave the highest total phenolic content and the uppermost antioxidant activity. Using the PCA, all extracts were clustered according to their respective groups with a total PC1 and PC2 of 95%. Based on the PLS analysis, the predicted functional groups that contributed to the antioxidant activity of *S. arvensis* leaf extracts were –OH and C–O, ascribing the phenolic compounds. Therefore, the FTIR-based fingerprinting with chemometrics could be used for clustering and characterizing the antioxidant activity of the *S. arvensis* leaf extracts.

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