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Original Research Article

The Effect of Extraction Method on Flavonoid Content and Antioxidant Activity of Red Betel and Green Betel Extracts

Nursamsiar Nursamsiar[1], Marwati Marwati[2], Syamsu Nur[1]

[1]*Department of Pharmaceutical Chemistry, Sekolah Tinggi Ilmu Farmasi Makassar, Indonesia, 90242*
[2]*Department of Pharmaceutical Biology, Sekolah Tinggi Ilmu Farmasi Makassar, Indonesia, 90242*

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ABSTRACT

Red betel (*Piper crocatum* Ruiz & Pav) and green betel (*Piper betle L*.) are traditionally used as medicines. Betel leaf contains flavonoid compounds with antihypertensive, antioxidant, and antibacterial potential. This study aimed to determine the effect of the extraction method on the total flavonoid content of the ethanol extract of red and green betel leaves using UV-Vis spectrophotometry. The samples were extracted by sonication, reflux, and Soxhlet methods, respectively, to obtain crude extracts of each species. The levels of flavonoids and their antioxidant activity were determined UV-Visible spectrophotometry. The results showed that the total flavonoid content of red betel leaves in the sonication, reflux, and Soxhlet methods were 11.4 ± 0.31, 16.1 ± 0.42, and 11.8 ± 0.05, respectively, and in green betel leaf, were10.9 ± 0.11, 6.4 ± 0.14, and 17.6 ± 0.09 mgEQ/g, respectively. The results of antioxidant activity testing using the DPPH, ABTS, and FRAP methods showed that green betel extract has significant antioxidant activity compared to red betel extract. Extraction of green betel leaves by reflux method gave the highest total flavonoid content and correlated with its activity as an antioxidant. This study can provide information regarding the effect of the extraction method on total flavonoid content and antioxidant activity of red and green betel extracts to serve as a basis for further research development.

Keywords: *Piper crocatum* Ruiz, *Piper betle*, Extraction Method, Sonication, Reflux, Soxhlet, flavonoids, antioxidants activity .

Introduction

Herbal medicinal plants are one of the essential elements in people's lives because of their various benefits, both as a source of food and for medicine. Although the efficacy of most of these plants has not been scientifically proven, as an alternative remedy for certain diseases, herbal plants have been used for generations through studies on traditional medicine and native knowledge of the population and provide the expected results in the healing process.[1]

Betel has long been known and used for generations to treat coughs, toothaches, fresheners, and so on.[2] Parts of the betel plant, such as roots, seeds, and leaves, have the potential for treatment, but the most commonly used are the leaves.[2,3] The benefits of betel leaf as a medicine are derived from its phytochemical components. A major phytoconstituent of betel leaf is flavonoid.[4]; other secondary metabolites of red betel are alkaloids, tannins, saponins, and triterpenoids. Flavonoids are differentiated into flavones, flavonols, flavanones, isoflavones, aurons, catechins, anthocyanidins, and chalcones.[3] In addition, red betel contains phenolic compounds in the form of chavicol, cavibetol acetate, and eugenol.[3,4] Green betel leaves contain several macro and micronutrients, including carbohydrates, fats, proteins, vitamins, and minerals.[2,3] Various activities of the nutrients and compounds in green betel leaves have been reported.[2] Thus, red and green betel plants have found significant uses in traditional medicine.

*Corresponding author. E-mail: nur.samsiar@stifa.ac.id
Tel: +6285315742087

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Flavonoids are secondary metabolite compounds with biological activities such as antiviral, antiallergic, lipid-lowering, antibacterial, and anti-inflammatory, preventing cardiovascular and cerebrovascular diseases.[5] Flavonoids are found in various plants and used as natural pigments and antioxidants in the food industry.[6] Flavonoids occur naturally as aglycones (O glycosides and C glycosides) in plants.[6,7] However, the stability of the aglycone correlates with low bioavailability, which limits its application in the food and pharmaceutical fields.[7] As such, there is a need for extraction methods that do not compromise the flavonoid compounds' stability.

Extraction methods can affect the levels of compounds such as flavonoids and some compounds with low stability [1,8–10]. Various extraction techniques are used to obtain bioactive components from plant components. There are conventional techniques, such as Soxhlet and reflux, to more advanced techniques, such as microwave and ultrasonic extraction. Depending on the study objective, choosing the best extraction method to obtain the highest biological activity in developing herbal medicines is necessary.[11] This study aimed to determine the effect of the extraction method on the flavonoid content profile of red and green betel leaves and their antioxidant activity, as well as to provide information on their chemical contents and bioactivity as an antioxidant for possible drug development.

Materials and Method

Materials

The materials include a micropipette (*Dragonlab Micropipette*) Bio red®, an analytical balance (Mettler toledo®), a reflux kit, a Soxhlet kit, a sonicator, a Shimadzu UV-Vis spectrophotometer (UV-1900) and a rotary evaporator (Buchi®). The reagents include ethanol (Merck®), ABTS (Sigma Aldrich®), $AlCl_3$ 10% (Sigma Aldrich®), DPPH (Sigma Aldrich®), 2,4,6-tripyridyl-s-triazine (Sigma Aldrich®), CH_3COONa (Sigma Aldrich®), $FeCl_3$ (Sigma Aldrich®), and quercetin (Sigma Aldrich®).

Plant collection and extraction
The leaves of red betel (*Piper crocatum* Ruiz & Pav) and green betel (*Piper betle L.*) were collected from Mocongloe, Maros Regency (5°08'08.0"S+119°31'53.6" E), South Sulawesi, Indonesia, in the third week of April 2022. The plants were identified by Dr. Andi Marnisa of the Plant Anatomy and Biology Laboratory, Universitas Negeri Makassar, South Sulawesi, Indonesia, with Specimen Number 121-23-SKAP.

Plant Extraction
Each dried plant sample was extracted by sonication, reflux, and Soxhlet methods. The extraction process for each of the methods is described below.

Reflux Extraction Method
The dried powders (100 g) of red and green betel leaves were weighed and extracted by reflux using 70% ethanol for 3 hours at 60ºC. The extract was filtered, and the filtrate was concentrated to dryness in a rotary evaporator (Rotavapor® R-300, Buchi Corporation, USA) to obtain a viscous red and green betel leaf extract. The percentage (%) extract yields were calculated.

Soxhlet Extraction Method
The dried powders (100 g) of red and green betel leaves were weighed and extracted by Soxhlet using 1L of 70% ethanol solvent. For exhaustive extraction, the process was repeated for 8 hrs. The filtrate resulting from the Soxhlet extraction was collected, filtered, and then concentrated using a rotary evaporator (Rotavapor® R-300, Buchi Corporation, USA) to obtain a thick extract. The percentage (%) extract yield was calculated.

Sonication Method
The dried powders (100 g) of red and green betel leaves were weighed and extracted by Soxhlet using 1L of 70% ethanol solvent by sonication (40 kHz) for 2 hours at 35 ℃. The extract was then filtered, and the filtrate was evaporated to dryness using a rotary evaporator to obtain a thick crude extract. The percentage (%) extract yield was then calculated.

Identification of flavonoid by specific reagent
The ethanol extracts (50 mg) of red and green betel were weighed and dissolved in a test tube with 2 mL of ethanol. A small amount of magnesium powder was added to the mixture, followed by drops of concentrated HCl. The appearance of yellow, brick red or orange indicates the presence of flavonoids.

Determination of Total Flavonoid Levels
The flavonoid content of red and green betel extracts was determined by UV-visible spectrophotometry. Quercetin was used as a standard for the calibration curve (2, 4, 6, 8, and 10 µg/mL) in determining total flavonoid content. Each standard solution concentration was mixed with 0.1 mL CH_3COONa (1 M) and 0.1 mL $AlCl_3$ (10% w/v) and allowed to stand for three minutes. The reaction mixture was made up to 5 mL with ethanol and allowed to stand for 30 minutes in a dark place at room temperature. The absorbance of the standard solutions was measured by a UV-Visible spectrophotometer at 430 nm. The absorbance values were plotted against concentration to generate the calibration curve equation. To determine the total flavonoid content of the extracts, the above procedure was repeated with 0.5 mL of a 1 mg/mL extract solution in place of quercetin. The total flavonoid content of each extract was expressed in mg, equivalent to quercetin per gram of dry extract (mgEQ/g). Tests for total flavonoid content in standard and samples were carried out in triplicate.[12,13]

Antioxidant Activity Testing
Anti-radical DPPH Assay
The extracts (10 mg each) from the various extraction methods were dissolved in 1 mL (1000 µg/mL) with ethanol Pro Analysis. DPPH solution (100 µL, 0.4 mM) was added into the microplate well, followed by 100 µL of various concentrations of each extract (31.25, 62.5, 125, 250, 500, and 1000 µg/mL). The mixture in the microplate was incubated for 30 minutes. After the incubation period, the absorbance of each sample was measured using a microplate reader at a wavelength of 515 nm.[14] A blank determination was carried out without the test samples or standard. The percentage inhibition of the DPPH free radical was calculated from the equation below:

$$\% \text{ Inhibition} = \frac{\text{Abs blank} - \text{Abs Sample}}{\text{Abs blank}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

The IC_{50} was computed from the linear regression equation y = a + bx derived from the plot of percent inhibition versus extract concentration, where x is the concentration (µg/mL), and y is the percent inhibition (%). Antioxidant activity is expressed by the 50% inhibitory concentration (IC_{50}), which is the sample concentration that can inhibit 50% of the DPPH radicals.

Anti-radical ABTS assay
Antioxidant activity was tested by making a concentration series of each sample solution (31.25, 62.5, 125, 250, 500, and 1000 µg/mL) from 100 µL of stock solution. Then, 1 mL of ABTS solution was added to each sample solution, and the volume was adjusted to 5 mL with ethanol pro analysis. The mixture was vortexed, and the absorption was measured with a UV-Vis spectrophotometer at 745 nm. The same procedure was done for the ascorbic acid and quercetin as standards.[15]
The IC_{50} value was calculated based on the percentage inhibition of ABTS radicals from each concentration of the sample solution using the equation below.

$$\% \text{ Inhibition} = \frac{\text{Abs blank} - \text{Abs Sample}}{\text{Abs blank}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The IC_{50} was computed from the linear regression equation y = a + bx derived from the plot of percent inhibition versus extract concentration, where x is the concentration (µg/mL), and y is the percent inhibition (%). Antioxidant activity is expressed by the 50% inhibitory concentration (IC_{50}), which is the sample concentration that can inhibit 50% of the ABTS radicals.

Ferric Reducing Antioxidant Power (FRAP) assay
The antioxidant activity of green and red betel leaf ethanol extracts was done using the FRAP method described by Nur et al. with slight modifications [14]. The stock solution was prepared by dissolving 10 mg of quercetin with distilled water, and the final volume was adjusted to 10 mL in a volumetric flask to obtain a concentration of 3.3 mM. 0.5 mL of each concentration 6, 8, 12, 18, 24, and 30 µM, quercetin was reacted with 2 mL of FRAP reagent in a ratio of 10:1:1 (acetate buffer: TPTZ: $FeCl_3$), and the volume was adjusted with distilled water to 5 mL. The mixtures were incubated at 37 C for 30 minutes. Then the absorption was measured with a UV-Vis spectrophotometer at 595 nm. The absorbance of each concentration series was plotted to obtain a standard curve equation (y = a + bx). The ferric-reducing antioxidant potential of 0.1% w/v extracts of the red and green betel was computed using the same procedure as the quercetin standard curve.

Statistical analysis
All the determinations were carried out in triplicates, and the results were expressed as mean+SD using Microsoft Excel 2019 version.

Results and Discussion

This study investigated the effect of various extraction methods on determining the total flavonoid content of red and green betel leaf extract using UV-Vis spectrophotometry to test the potential antioxidant activity of the ethanol extract of red and green betel leaves using DPPH and FRAP antioxidant assays. The samples used in this study were red and green betel leaf extracts collected from Moncongloe District, Maros City, South Sulawesi Province, extracted using 70% ethanol by three extraction techniques. Flavonoids are generally more soluble in water or polar solvents like ethanol.[8,9]
Tables 1 and 2 show that the highest percentage of extract yield for the two samples was obtained using the reflux method, red betel leaf (7.11%) and green betel leaf (6.24%). These results indicate that the phytochemicals present in the two samples are more effectively

extracted with increasing temperatures than the ambient conditions at room temperature. This is affected because, in hot conditions during the extraction process, cell wall and membrane damage occur due to the pressure difference between inside and outside the cell.[10] Secondary metabolites contained in the cytoplasm of plants will dissolve into organic solvents during the extraction process with the effect of increasing temperature. However, an increase in temperature that is too high such as the Soxhlet method can cause damage to thermolabile secondary metabolites.[11,16]

The extracts obtained were subjected to phytochemical screening to identify the presence of flavonoids as orange colour precipitates. The results showed that red and green betel leaf ethanol extract positively contained flavonoids (Table 3). The addition of Mg metal and concentrated HCl in the flavonoid structure identification screening aims to reduce the nucleus of the benzopyrone present in the flavonoid structure so that a colour change from yellow or orange to red can occur. Adding an HCl reagent resulted in an oxidation-reduction reaction between the Mg metal as a reducing factor and flavonoid compounds.[17] Flavonoids in samples are quantified spectrophotometrically by the aluminium chloride assay. The analysis results were expressed in quercetin equivalents for each gram of dry sample. Flavonoids are phenolic derivatives with conjugated aromatic ring systems that absorb UV light. Flavonoid content in plants is determined using standard quercetin, with a ketone group in the C-4 and a hydroxyl group in the C-3 and C-5 atoms, and related to flavones and flavanols.[12] In determining the levels of flavonoids in the sample solution, $AlCl_3$ was added to form a coloured complex due to a reduction-oxidation reaction between $AlCl_3$ and the hydroxyl and carbonyl groups in flavonoid compounds which is determined colourimetrically. The complexation between the flavonoid compounds and the $AlCl_3$ reagent causes a shift in the wavelength to the visible direction, indicated by the solution producing a deep yellow colour.[12,18,19]

Table 1: Percentage extract yields of Red Betel Leaf Extract

No	Sample	Sample weight (g)	Extract (g)	% Yield
1	Sonicator method	100 g	4.25	4.25%
2	Reflux method	100 g	7.10	7.1%
3	Soxhlet method	100 g	5.12	5.12%

Table 2: Percentage extract yields Green Betel Leaf Extract

No	Sample	Sample weight (g)	Extract (g)	% Yield
1	Sonicator method	100 g	3.00	3%
2	Reflux method	100 g	6.24	6.24%
3	Soxhlet method	100 g	4.72	4.72%

The results in Table 4 shows that the ethanol extract of red betel leaves has the highest levels of flavonoids in the reflux method in green betel (16.1 mgEQ/g extract) and 17. 4 mgEQ/g extract in the Soxhlet method. The results of the analysis on red betel showed that the total flavonoid content in the extract resulting from reflux was greater than the results of Soxhlet and ultrasonic methods. This is probably due to the heating process in the reflux method, which increases the solvent's ability to extract insoluble compounds at room temperature, resulting in the maximum extraction of compounds.[10] Whereas in green betel, the results obtained were inversely proportional to those obtained with the Soxhlet methods, which showed the highest levels of flavonoids.

According to research, the temperature and length of heating time in the extraction process can affect the levels of compounds obtained. In general, the extracted active substance's solubility increases as temperature rises. In this study, the soxhlet and reflux methods use higher temperatures than the ultrasonic method. Flavonoids are compounds that are stable to heating at a specific temperature. The extraction process carried out hot helps release flavonoid compounds in the material, contributing to increased amounts of flavonoids. Extraction time also affects the levels of the extracted active ingredients. The appropriate extraction time produces ideal amounts of substances, whereas an extraction period that is too brief allows only some of the active chemicals in the material to be extracted. The greater the period of the solute in contact with the solvent, the more chemical components extracted.[9–11]

The antioxidant activity of the ethanol extract of red and green betel leaves was evaluated using the DPPH, ABTS, and FRAP methods. The selection of the antioxidant activity test method is representative of evaluating the antioxidant potential of the ethanol extract of red and green betel leaves using the Hydrogen Atoms Transport (HAT) and Single Electron Transport (SET) mechanisms, including DPPH and ABTS method and redox mechanism in FRAP method.[20,21] The antioxidant potential of a sample is evaluated by determining the IC_{50} value or the potential of an antioxidant agent in reducing free radicals by 50%.[20,21] This can be used as a reference in determining the potential antioxidant activity of the ethanol extract of red and green betel leaves. The results (Figure 1) of the antioxidant activity screening in the DPPH (A) and ABTS (B) assays showed a linear correlation of antioxidant activity with increasing concentrations in red and green betel leaf extract samples. This is directly proportional to flavonoid compounds in red and green betel leaf extract (Table 4). In the DPPH assay, compounds that have the property of donating hydrogen atoms or electrons to the DPPH radicals can be assessed by a colour change from the violet DPPH colour to the stable yellow DPPH.[15,22] According to the findings in Table 5, the ethanol extract of red and green betel leaves showed weak antioxidant activity with an IC_{50} value > 200 µg/mL. The results obtained indicate that compounds with the strongest antioxidant capacity in scavenging DPPH radicals are more soluble in semi-polar solvents.[21]

In the ABTS radical assay, the ethanol extract of red and green betel leaves in the presence of potassium persulfate oxidizes the ABTS into cationic radicals during the incubation period, changing colour to bluish-green. Potential activity in reducing ABTS cation radicals is shown from the colour changing to faded green. The higher the concentration of the test compound, the more faded the colour of the ABTS radicals.[22,23] The result of the antioxidant activity in the ABTS cation radicals reduction (Table 5), the ethanol extract of red and green betel leaves (Table 5), obtained by reflux extraction showed strong antioxidant activity (IC_{50} value of 19.71 µg/mL) compared to the other extracts obtained through other methods. This is supported by the content of total flavonoid compounds listed in Table 4 and the presence of compounds from the results of the phytochemical screening conducted,[24] which supports a synergistic effect in reducing ABTS cation radicals. According to research conducted by Gulcin,[23] ABTS cation radicals are more reactive than DPPH radicals which not only scavenge free radicals with the HAT (Hydrogen Atom Transport) mechanism but also involve the SET (Single Electron Transport) mechanism in scavenging ABTS cation radicals.

The FRAP method of measuring antioxidant activity is based on the ET (Electron Transport) mechanism, which measures the reduction of ferric ion (Fe^{3+})-ligand complexes to highly blue ferrous ion (Fe^{2+}) complexes by antioxidants in an acidic medium. In other words, this measurement method is based on the ability of antioxidants to reduce the iron complex 2,4,6-trippyridyl-s-triazine $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ to a dark blue iron complex $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ in acidic medium.[23] According to the findings of the FRAP method's antioxidant activity tests (Table 5), the ethanol extract of betel leaves obtained by the reflux method exhibited a better ferric ion-reducing power of 33.76 µM/g. This shows that the ethanol extracts of red and green betel leaves have the potential to reduce Fe^{3+} ions, preventing free radical generation during the Fenton reaction.[25–27] Increasing the content of flavonoid and phenolic

compounds contained in red and green betel leaf extracts has a better correlation with antioxidant activity in reducing iron.[21,28]

Conclusion

The results of the study showed that the average total flavonoid content of the ethanol extract of red betel leaves was higher in the extract obtained by the reflux method (16.1±0.42 mgEQ/g), while the total flavonoid content obtained in green betel leaves was highest in the Soxhlet extraction method (17.6±0.09 mgEQ/g). This study also showed that the ethanol extract of green betel obtained through the Soxhlet extraction method exhibited the most significant antioxidant activity compared to red betel in scavenging DPPH radicals, ABTS, and iron reduction. This study provides an overview of the profile of total flavonoid content and antioxidant activity of red and green betel obtained from different extraction methods. In future studies, researchers may make informed decisions from these results to improve flavonoid yields.

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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Table 3: Qualitative test results of red and green betel leaf extracts with various extraction methods

Sample	Extraction Method	Reagent	Result
Ethanol Extract *Piper Crocatum*	Sonicator	magnesium powder concentrated HCl	+
	Reflux		+
	Soxhlet		+
Ethanol Extract *Piper betle*	Sonicator		+
	Reflux		+
	Soxhlet		+

Table 4: Results of the total flavonoid content of the ethanol extract of red and green betel leaves with various extraction methods

Sample	Extraction Method	Flavonoid Content (mgEQ/g extract)	Standard Deviation
Piper crocatum	Sonicator	11.4	0.31
	Soxhlet	11.8	0.05
	Reflux	16.1	0.42
Piper betle	Sonicator	10.9	0.11
	Soxhlet	17.6	0.09
	Reflux	6.4	0.14

Table 5: Test results for the antioxidant activity of the ethanol extract of red and green betel leaves

Sample	Antioxidant Activity		
	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP µM/g extract
SMUS (Sonicator *Piper crocatum)*	441.76 ± 4.03	495.29 ± 3.22	0.53 ± 0.07
SMREF (Reflux *Piper crocatum*)	527.238 ± 8.29	157.48 ± 1.83	2.4 ± 0.62
SMSOX (Soxhlet *Piper crocatum*)	268.93 ± 4.92	543.94 ± 5.02	2.33 ± 0.09
SHUS (Sonicator *Piper betle*)	273.49 ± 1.87	20.67 ± 0.98	20.93 ± 0.99
SHREF (Reflux *Piper betle*)	260.73 ± 2.45	19.71 ± 1.21	33.76 ± 1.76
SHSOX (Soxhlet *Piper betle*)	454.09 ± 3.30	36.85 ± 0.79	25.93 ± 1.45

Note: SMUS (Red betel extract by sonication method), SMREF (Red betel extract by reflux method), SMSOX (Red betel extract by soxhlet method), SHUS (green betel extract by sonication method), SHREF (green betel extract by reflux method), and SHSOX (green betel extract by soxhlet method)

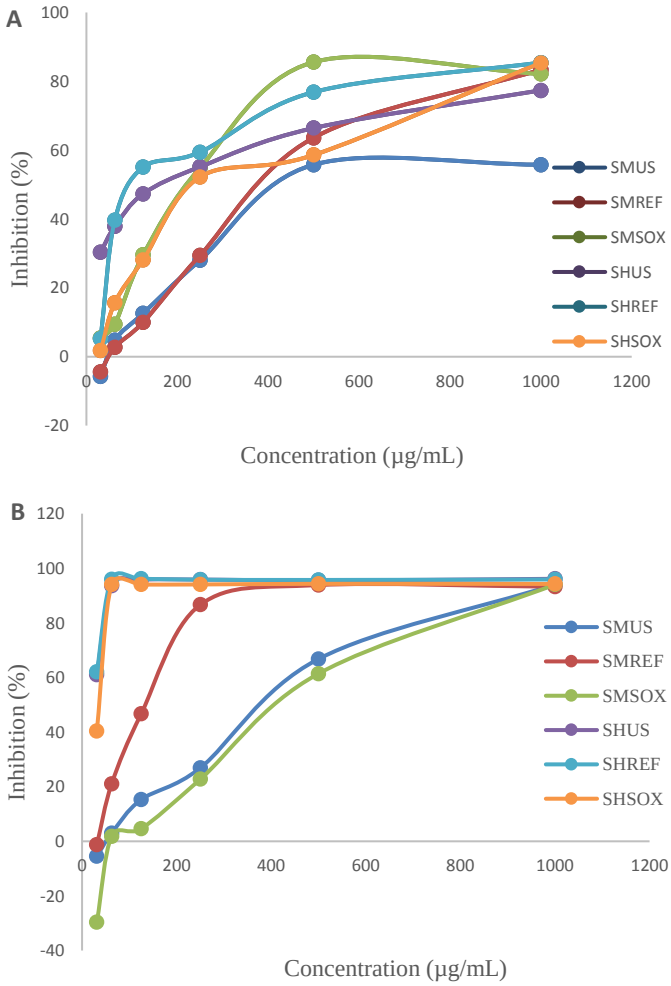

Figure 1: Inhibition (%) profile of each sample along with an increasing concentration in absorbing (A) DPPH radicals and (B) ABTS radicals. SMUS (Red betel extract by sonication method), SMREF (Red betel extract by reflux method), SMSOX (Red betel extract by soxhlet method), SHUS (green betel extract by sonication method), SHREF (green betel extract by reflux method), and SHSOX (green betel extract by soxhlet method)

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