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Efficacy of Biological Activity of *Andrographis Paniculata* Extracted by using Supercritical Carbon Dioxide (Sc-CO_2) Extraction

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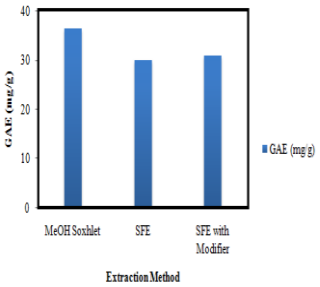
Graphical abstract



Abstract

Andrographis paniculata plant has been widely used for traditional medicine such as curing fever, diarrhoea, and inflammation. In this study, the main component (Andrographolide) is extracted from the leaves of *A.* paniculata using supercritical carbon dioxide (Sc-CO_2) and Sc-CO_2 assisted by methanol as a modifier solvent. The methanol soxhlet extraction as a standard method was used to compare with the Sc-CO_2 extraction. This work also focuses on the determination of anti-oxidant activity of Sc-CO_2 extraction extracts of *A. paniculata* plant. The Sc-CO2 extracts of *A. paniculata* for both Sc-CO_2 extractions with and without modifier showed promising antioxidant activity. Free radical scavenging potential of both Sc-CO_2 extractions of *A. paniculata* plant was evaluated by using reducing power. In this method, ascorbic acid was used as a standard for determining reducing power. Both Sc-CO_2 extract of *A. paniculata* plant exhibited appreciable activity as compared to the methanol soxhlet extract, indicating that *A. paniculata* has promising free radical scavenging activity.

Keywords: Modifier; soxhlet; supercritical fluid; anti-oxidant activity

1.0 INTRODUCTION

Andrographis paniculata, locally known as Hempedu Bumi grows widely in the tropical areas of South East Asia, India and China with annual growth of 30–70 cm height. In Malaysia, this plant has been extensively used for traditional medicine and help against fever, dysentery, diarrhea, inflammation, and sore throat. Furthermore, it is a promising new way for the treatment of many diseases, including HIV, AIDS, and numerous symptoms associated with immune disorders [1]. There are three (3) main diterpenoid lactones identified in the *A. paniculata* leaves were andrographolide, neo-andrographolide and deoxyandrographolide [2-4]. Andrographolide, which is grouped as an unsaturated trihydroxy lactone has molecular formula of $C_{20}H_{30}O_5$. Andrographolide is easily dissolved in methanol, ethanol, pyridine, acetic acid and acetone, but only slightly dissolved in ether and water. The melting point of this substance is 228–230°C, while its ultraviolet spectrum in ethanol, λmax is 223 nm [3].

Extraction using organic solvent is one of the most common method of separating bioactive compounds or components from their natural hosts. But, because of these liquid solvents are not easy and usually not able to be completely removed by the existing separation techniques and their traces may remain present in the final product. Besides that, the extraction using only organic solvent is no longer attractive from clinical, environmental, energy and time consumption point of views [5]. Therefore, supercritical fluid extraction (SFE) is a better alternative method to extract bioactive components from their natural hosts, since this method offers shorter extraction times, cheaper operating cost, higher extraction selectivity, safer condition (non toxic, non flammable, non hazardous) and adjustable solvating power [6]. SFE also have been known as Supercritical Carbon Dioxide (Sc-CO_2).

Although Sc-CO_2 is only capable of dissolving non-polar solutes but with respect to such attractive properties of CO_2, extraction of highly polar analytes has usually been carried out by using carbon dioxide (CO_2) containing a few percent of organic modifier solvent [7-8]. The presence of modifier in supercritical fluid will lead to higher extraction efficiencies and has been shown to significantly improve the solubility of relatively polar solutes. [8]. Common modifiers used are methanol, ethanol, propanol, etc. [6]. In general a modifier that has a lower critical temperature than the supercritical gas causes a decrease in the solubility of a low volatile analyte in gas, whereas a modifier of higher critical temperature causes an increase in solubility.

Although several studies about supercritical fluid extraction, SFE have been carried out but still lack articles reported about the supercritical extraction of *A. paniculata* leaves. Therefore this experiment has been done to observe

potential of SFE and the efficacy biological activity of *A. paniculata* extracted by using SFE.

2.0 EXPERIMENTAL

2.1 Sample Collection

Dried ground *A. paniculata* were obtained locally from Centre of Lipid & Applied Research (CLEAR), Universiti Teknologi Malaysia were then sealed up in an airtight container and stored in the freezer at temperature -20°C until further used.

2.2 Preparation of Extract

2.2.1 Soxhlet Extraction

In this study the Soxhlet extraction method was used as an extraction reference to observing the efficacy and performance of SFE extraction.

For Soxhlet extraction, the sample was weighted about 20 g of *A. Paniculata* powder with 3 mm of particle size, then the sample was placed inside a cellulose thimble. The soxhlet extractor then was placed onto the flask containing selected solvent (400 mL) for the experimental work. The soxhlet extractor were equipped and installed with the condenser. Solvent was heated to reflux and the temperature was set based on its boiling point. Vaporized solvent went up through distillation arm and condensed back to the extraction thimble. On the other hand, the extraction condition used for soxhlet extraction method were temperature at 60°C, operated at ambient temperature for 6 hours of extraction regime and methanol was used as an extraction solvent.

Excess solvent was removed by using rotary evaporator to obtain the essential oil. The temperature of the rotary evaporator was set according to the boiling point of the solvent. Besides that, the speed of the rotary evaporator was set approximately 70 rotations per minute, rpm.

2.2.2 Supercritical Carbon Dioxide (Sc-CO_2) Extraction

SFE extractions were performed using a designated Supercritical Fluid Extraction at Center of Lipid and Applied Research (CLEAR), UTM. Extraction of andrographolide was conducted using two (2) different extraction methods of SFE (SFE without modifier and SFE assisted with modifier). For SFE the conditions used were temperature (60°C), pressure (30 MPa) and solvent flow rates (4 mL/min) with Static-Dynamic extraction mode (1 hour static and 30 minutes dynamic) and 10 g of sample (3 mm of particle size).

In SFE extraction, the weighted sample was placed into the stainless steel extraction cell and for SFE extraction assisted with modifier solvent was added up with 5 mL of methanol as modifier solvent. The extraction cell was then placed in the extraction chamber. The Supercritical Fluid Extraction (SFE) conditions were set based on extraction condition above. Apparatus used were constant flow Lab Alliance CO_2 pump connected with CO_2 tank equipped with stainless steel extraction placed in the oven (Memmert) with restrictor valve and back pressure regulator (Jasco).

2.3 Biological Activity

2.3.1 Total Phenolic Content

The total phenolic content of the sample was measured based on the methods described by Singleton *et al.* [9] with slight modification. Briefly, an aliquot of 0.1 ml extract was mixed with 1.0 ml Folin-Ciocalteau phenol reagent (Sigma-aldrich Co., St. Louis, MO, USA) and allowed to react for 3 min. Then a 300 µL of 1N Na_2CO_3 (Riedel-deHaen, Seelz, Germany) was added and allowed to react for 90 min at room temperature comparatively to gallic acid standard. Absorbance was measured at 725 nm using an automated microplate reader (Multiskan GO). The experiment was repeated thrice and the result was expressed as mg of gallic acid/g extract.

2.3.2 Total Flavanoid Content

The total flavonoid of the sample was determined using a modified colorimetric method described previously by Zhishen *et al*. [10] and used quacertin (Sigma-Aldrich Co.) as a standard. Extracts or standard solutions (250 µL) were mixed with distilled water (1.25 ml) and 5% NaNO2 (75 µL). After standing for 6 minutes, the mixture was combined with 10% AlCl3 solution (150 µL). 1 M NaOH (0.5 mL) and distilled water (275 µL) was added to the mixture 5 minutes later. The absorbance of the solutions at 510 nm was then measured. The experiment was repeated thrice and the results were expressed as mg of quacertin/g extract.

2.3.3 Scavenging Effect on 2,2-Diphenyl-1-Picrylhydrazil Radical (DPPH)

The radical scavenging ability of essential oil was determined as described by Mensor *et al*. [11]. Briefly, one ml from 5 mg/L alcohol solution of DPPH was added to 2.5 mL of extract. The samples were kept at room temperature in the dark and after 30 min the optic density was measured at 518 nm using UV-VIS. The antiradical activity (AA) was determined by the following formula:

$$\text{Percentage of inhibition} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

2.3.4 Statistical Analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses ± standard error. The magnitude of correlation between variables was done using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. XII, 2004).

3.0 RESULTS AND DISCUSSION

Methanol, SFE and SFE assisted with modifier extracts were prepared to examine the total phenolic content, flavanoid concentration and antioxidant activity. The percentage of yield of extract obtained from dry plant material was measured for each extract (Table 1). The highest yield of solid residue was obtained using methanol soxhlet extraction and followed by SFE assisted with modifier and SFE without modifier.

Table 1 The percentage of yields of solid residue of *A. paniculata* after extraction

Extraction method	Percentage of Yield (%)
MeOH Soxhlet	6.70
SFE	0.29
SFE with Modifier	1.41

The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: y = 2.334x – 0.012, $R^2 = 0.992$).

The values obtained for the concentration of total phenols are expressed as mg of GAE/g of extract (Table 2). The total phenolic contents in the examined extracts ranged from 30.04 to 36.56 mg GAE/g. The highest concentration of phenols was measured in methanolic extract and followed by SFE assisted with modifier then lastly SFE without modifier. Based on Table 2 it showed there are significant different between methanolic extract to the both types of SFE extract. The total phenolic content in plant extracts of the species *A. paniculata* depends on the type of extract such as the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction [12].

The concentration of flavonoids in various plant extracts of the species *A. paniculata* was determined using spectrophotometric method with aluminum chloride ($AlCl_3$). The content of flavanoids was expressed in terms of quacertin equivalent, QE (the standard curve equation: y = 0.393x – 0.034, R2 = 0.998), mg of QE/g of extract (Table 2). The concentration of flavonoids in plant extracts from A. Panicullata ranged from 154.13 to 179.81 mg of QE/g. SFE without modifier extracts contains the highest flavanoid concentration (179.81 mg QE/g) and followed by methanolic and SFE assisted with modifier with 162.00 and 154.13 mg of QE/g respectively. The concentration of flavanoids in plant extracts depends on the polarity of solvents used in the extract preparation (Min and Chun Zhao, 2005).

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Table 2 Total phenolic content (TPC) and total flavonoid content (TFC) of *A. paniculata*

Extraction method	TPC (mg/g)	TFC (mg/g)
MeOH Soxhlet	36.56^b S.E ± 0.082	162.00^a S.E ± 0.848
SFE	30.04^a S.E ± 0.289	179.81^b S.E ± 0.848
SFE with Modifier	30.99^a S.E ± 0.378	154.13^a S.E ± 4.576

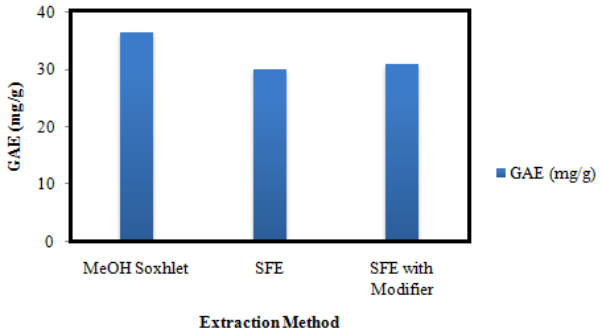

Figure 1 Comparison of TPC in gram gallic acid equivalents (GAE) per g extract in *A. paniculata*

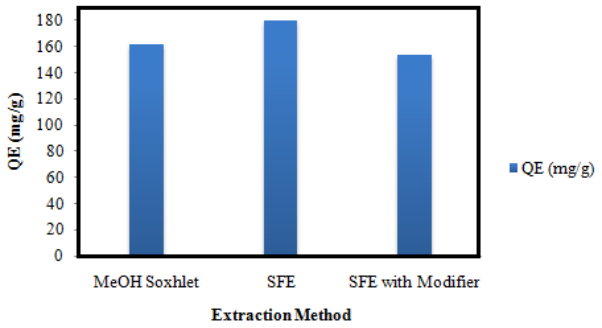

Figure 2 Comparison of TFC in gram quacertin equivalents (QE) per g extract in *A. paniculata*

The antioxidant activity of different plant extracts from *A. Paniculata* was determined using a methanol solution of DPPH reagent. DPPH is very stable free radical. Unlike in vitro generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 510 nm. This purple colour generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colourless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 510 nm band [14].

The antioxidant activity of 3 different extracts from the species *A. paniculata* is expressed in terms of percentage of inhibition (%) and IC50 values (µg/ml) (Figure 3). Parallel to examination of the antioxidant activity of plant extracts, the values for two standard compounds were obtained and compared to the values of the antioxidant activity. The standard substance was ascorbic acid.

The examination of antioxidant activities of plant extracts from *A. paniculata* showed different values. The obtained values varied from 90.09% to 136.42%. The largest capacity to neutralize DPPH radicals was found for methanolic soxhlet extract, which neutralized 50% of free radicals at the concentration of 163.64 µg/mL and followed by SFE assisted with modifier (242.03 µg/mL) and SFE without modifier (341.51 µg/mL). In comparison to IC50 values of quacertin, methanolic extract from *A. paniculata* manifested the strongest capacity for neutralization of DPPH radicals and also shows that both SFE extraction have a great potential.

The extraction of antioxidant substances of different chemical structure was achieved using different extraction method and based on few study qualitative composition of plant extracts revealed the presence of high concentrations of phenols in the extracts obtained using polar solvents. The extracts that perform the highest antioxidant activity (Figure 3) have the highest concentration of phenols (Table 2). Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may contribute directly to their antioxidant action [15].

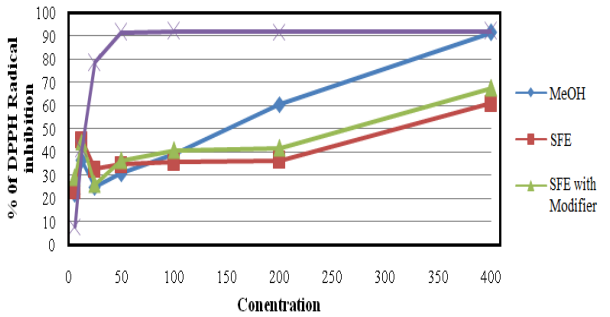

Figure 3 Antioxidant (DPPH scavenging) activity of investigated plant extracts presented as percentage of DPPH radicals inhibition and IC50 values (µg/ml)

Flavonoids are class of secondary plant metabolites with significant antioxidant and chelating properties. Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups [16]. Therefore, this showed that all methanolic soxhlet, SFE without modifier and SFE assisted with modifier extracts from *A. paniculata* have high concentration of total phenols (Figure 1) and flavonoids (Figure 2).

■4.0 CONCLUSION

Results of the study suggest the great value of the species *A. paniculata* for use in pharmacy and phytotherapy. Based on this information, it could be concluded that this plant is natural sources of antioxidant substances of high importance.

It is noticed that the highest concentration of phenolic compounds in the extracts also could be obtained by using SFE technique. Other than methanol soxhlet extraction, the SFE extract also manifested greater power of extraction for phenolic compounds from *A. paniculata*.

Further studies of this plant species and SFE extraction technique should be directed to carry out in optimization and in-vivo studies of its medicinal active components in order to prepare a natural pharmaceutical product of high value and more environmental friendly.

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